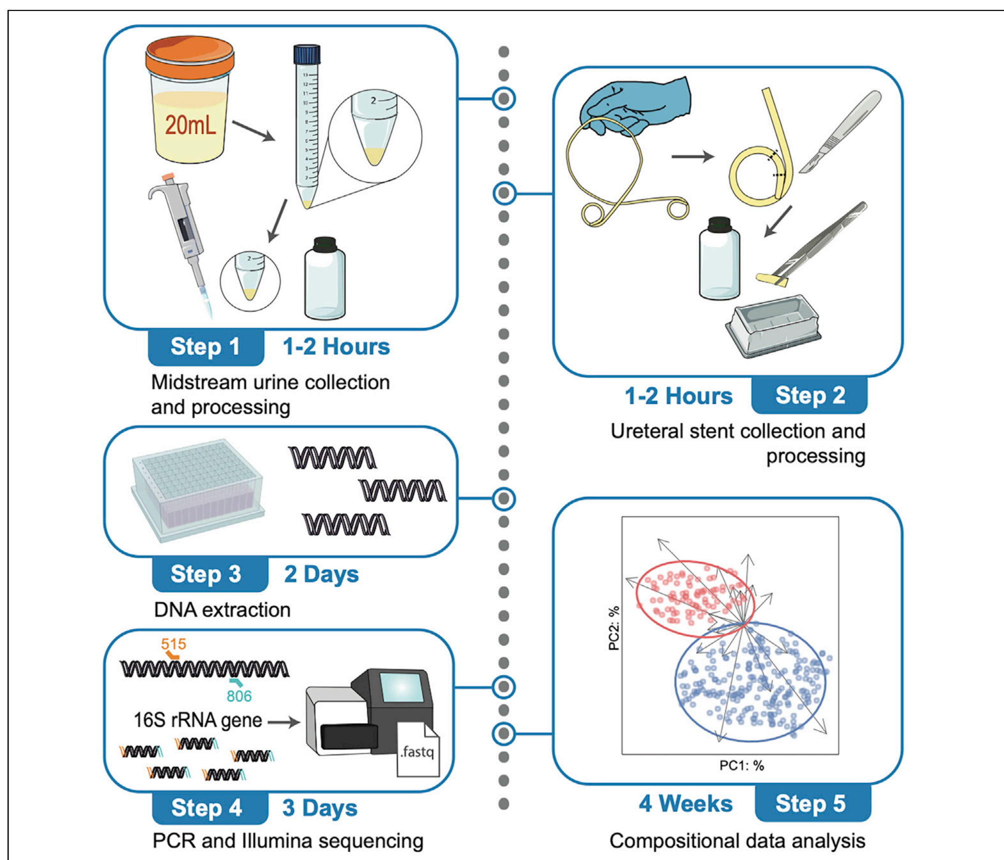
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Processing human urine and ureteral stents for 16S rRNA amplicon sequencing



Ureteral stents are commonly used medical devices that harbor a unique and patient-specific microbial community. This protocol describes an optimized procedure for high-quality DNA extraction from both urine and ureteral stent samples for the purpose of downstream microbiota characterization by amplicon sequencing. Detailed instruction is provided for 16S rRNA gene V4 region sequencing with the Illumina platform, which enables accurate and reproducible microbiota profiling of low bacterial abundance urine and stent samples.

Kait F. Al, Jeremy P. Burton

kal@uwo.ca (K.F.A.)
Jeremy.burton@lawsonresearch.com (J.P.B.)

Highlights

The urinary tract harbors low-abundance microbiota capable of influencing health

Traditional sample preparation yields variable results vulnerable to contamination

Protocol optimizes urological specimen processing for 16S rRNA amplicon sequencing

The method ensures high-quality, reproducible urological microbiota data generation

Al & Burton, STAR Protocols 2, 100435
June 18, 2021 © 2021 The Authors.
https://doi.org/10.1016/j.xpro.2021.100435

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Processing human urine and ureteral stents for 16S rRNA amplicon sequencing

Kait F. Al[1,2,4,*] and Jeremy P. Burton[1,2,3,5,*]

[1]Centre for Human Microbiome and Probiotic Research, Lawson Health Research Institute, London, ON, Canada

[2]Department of Microbiology and Immunology, The University of Western Ontario, London, ON, Canada

[3]Division of Urology, Department of Surgery, The University of Western Ontario, London, ON, Canada

[4]Technical contact

[5]Lead contact

*Correspondence: kal@uwo.ca (K.F.A.), Jeremy.burton@lawsonresearch.com (J.P.B.)
https://doi.org/10.1016/j.xpro.2021.100435

SUMMARY

Ureteral stents are commonly used medical devices that harbor a unique and patient-specific microbial community. This protocol describes an optimized procedure for high-quality DNA extraction from both urine and ureteral stent samples for the purpose of downstream microbiota characterization by amplicon sequencing. Detailed instruction is provided for 16S rRNA gene V4 region sequencing with the Illumina platform, which enables accurate and reproducible microbiota profiling of low bacterial abundance urine and stent samples.
For complete details on the use and execution of this protocol, please refer to Al et al. (2020).

BEFORE YOU BEGIN

Preparation of positive controls

Timing: 1 day

1. Prepare two separate 15 mL conical centrifuge tubes, each containing 10 mL LB broth. Inoculate one tube with a single colony of *Staphylococcus aureus* Newman, and the other with a single colony of *Escherichia coli* DH5α. Incubate aerobically for 12 h at 37°C.
2. After growth, dispense the cultures into 100 μL aliquots with a sterile filter pipette tip into labelled 1.5 mL microfuge tubes.
3. Freeze the aliquots at −20°C until DNA extraction.

Note: Commercially available defined microbial communities can also act as positive controls to assess the bias and potential contamination in the downstream workflow. An example would be the ZymoBIOMICS standards (Zymo Research).

Additional protocol preparation

Timing: 30 min

1. See Materials and Equipment for preparation of necessary materials
2. Set centrifuge to 20°C and decontaminate with RNase AWAY™
3. Obtain fresh mid-stream urine and ureteral stent samples from individuals meeting the study-specific inclusion and exclusion criteria (Table 1).

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Table 1. Example inclusion and exclusion criteria for study participation

Inclusion criteria	Exclusion criteria
Male or Female At least 18 years of age Has an indwelling ureteral stent scheduled for removal Able to provide a clean-catch midstream urine sample Able and willing to provide informed consent	In the opinion of the treating urologist, it is not in the patient's best interest to participate

⚠ CRITICAL: Human urine and ureteral stent samples should be obtained using an Institutional Review Board (IRB) approved protocol. Human samples are considered to be potentially infectious and should be handled in Biosafety Level II cabinets using standard aseptic precautions.

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
Escherichia coli DH5α	ATCC	ATCC 68233
Staphylococcus aureus Newman	ATCC	ATCC 25904
Biological samples		
Mid-stream urine (20 – 50 mL)	Human	Health Sciences Research Ethics Board at the University of Western Ontario (REB #107941)
Ureteral stent	Human	Health Sciences Research Ethics Board at the University of Western Ontario (REB #107941)
Chemicals, peptide, and recombinant proteins		
LB broth	BD Difco	Catalog No. B244601
RNase AWAY	Thermo Scientific	Catalog No. 7003PK
Nuclease free water	Ambion	Catalog No. AM9932
GoTaq Hot start Colorless Master Mix	Promega	Catalog No. M5133
Ethanol, 99%	Fisher Chemical	Catalog No. BPA9954
Critical commercial assays		
DNEasy PowerSoil HTP 96 Kit	QIAGEN	Catalog No. 12955-4
Quant-iT PicoGreen dsDNA assay	Invitrogen	Catalog No. P11496
QIAquick PCR Purification Kit	QIAGEN	Catalog No. 28106
MiSeq Reagent Kit v3 (600-cycle)	Illumina	Catalog No. MS-102-3003
PhiX Control v3	Illumina	Catalog No. FC-110-3001
Deposited data		
Raw data	This paper and (Al et al., 2020)	16S rRNA sequence data (NCBI) BioProject ID #PRJNA601180
Oligonucleotides		
16S rRNA forward primer 515F: GTGCCAGCMGCCGCGGTAA	(Caporaso et al., 2012)	V4EMB forward
16S rRNA reverse primer 806R: GGACTACHVGGGTWTCTAAT	(Caporaso et al., 2012)	V4EMB reverse
Software and algorithms		
FastQC	(Andrews, 2010)	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
DADA2 v1.14	(Callahan et al., 2016)	https://benjjneb.github.io/dada2/
SILVA database v132	(Quast et al., 2013)	https://www.arb-silva.de
R v3.6.1	R Core Team	https://www.r-project.org
CoDaSeq v0.99.4	(Gloor and Reid, 2016)	https://github.com/ggloor/CoDaSeq

(*Continued on next page*)

Continued

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
ALDEx2 v1.11.0	(Fernandes et al., 2013)	https://bioconductor.org/packages/release/bioc/html/ALDEx2.html
Vegan v2.5-6	(Oksanen et al., 2019)	https://cran.r-project.org/web/packages/vegan/vegan.pdf
MaAsLin2 v1.1.1	(Mallick et al., 2020)	http://huttenhower.sph.harvard.edu/maaslin2
GraphPad Prism v8.3.1	GraphPad Software	N/A
Other		
60 mL Urine collection container with screw cap (sterile)	Starplex Scientific Inc.	Catalog No. B602-1O
50 mL Polypropylene centrifuge tubes (sterile)	Corning	Catalog No. C352070
1.5 mL Sterile microcentrifuge tube with screw caps	Fisherbrand	Catalog No. 515SFIS
1.5 mL Sterile microcentrifuge tube	Axygen	Catalog No. 022364111
Microcentrifuge	Thermo Scientific	Catalog No. 75002440
Razor blade	Fisherbrand	Catalog No. 270100
100 mL Sterile reservoir	Axygen	Catalog No. RESV100SI
Forceps	Fisherbrand	Catalog No. 12-000-157
200 µL PCR-grade filter tips	Invitrogen	Catalog No. LSAM12655
1250 µL PCR-grade filter tips	Eppendorf	Catalog No. 0030078594
50–1200 µL Electronic pipette	Eppendorf	Catalog No. 4861000163
Adhesive PCR plate foils	Thermo Scientific	Catalog No. AB0626
Biomek Automated Workstation	Beckman Coulter	Catalog No. 986120
96-well PCR microplate	Axygen	Catalog No. PCR96M2HSC
Liquid handler 20 µL pipette tips	Thermo Scientific	Catalog No. 91802105
Liquid handler 130 µL pipette tips	Thermo Scientific	Catalog No. 919021
96-well plate PCR thermal cycler	Eppendorf	Catalog No. 6333000022
96-well plate shaker	QIAGEN	Catalog No. 85300
96-well shaker adapter plates	QIAGEN	Catalog No. 11990
25 mL serological pipette	Fisherbrand	Catalog No. 170357N
Fluorescent microplate reader	Molecular Devices	Catalog No. SpectraMax M5
Pipet filler	Thermo Scientific	Catalog No. 9501
96-well plate centrifuge	Eppendorf	Catalog No. 022623508
Deepwell plate centrifuge rotor	Eppendorf	Catalog No. 22638564
Biomek reservoir 40 mL	Beckman Coulter	Catalog No. 534680
Qubit fluorometer	Thermo Scientific	Catalog No. Q33238

⚠ CRITICAL: Observe caution and wear gloves and eye protection when handling ethanol. Ethanol is a flammable, toxic chemical that can cause irritation upon inhalation or skin contact.

STEP-BY-STEP METHOD DETAILS

Note: Unless otherwise noted, all steps should be performed at a temperature between 20 and 25°C.

Urine and stent sample pre-storage preparation

⏲ Timing: 20 min

Human urine and stent samples must be partially processed within 4–6 h of collection prior to their frozen storage in order to optimize downstream DNA yield (Jung et al., 2019, Bundgaard-Nielsen et al., 2020). Importantly, urine samples should not be refrigerated prior to their processing due to increased precipitation of urinary sediments, which has been shown to decrease DNA yield

Table 2. Classification of stent encrustation

Grade of encrustation	Visual characteristics
0	Like-new
1	Discoloration only
2	Mild encrustation (≤1 mm thick)
3	Heavy encrustation (>1 mm thick)

(Ribeiro et al., 2013, Ackerman et al., 2019). DNA extraction should be performed together once all samples are collected to minimize batch effects.

1. Mid-stream urine sample preparation
   a. A volume of 20 mL or greater of mid-stream urine should be collected into a urine collection container by the study participant prior to the stent removal procedure.

   Note: Mid-stream urine is more representative of the bladder microbiota, rather than a first-void urine sample which can closely resemble the periurethral microbiota (Karstens et al., 2018). Study participants should be instructed on how to properly perform a clean-catch sample collection, or be provided with a collection device intended for isolation of mid-stream urine (Southworth et al., 2019).

   b. With a sterile serological pipette, transfer 20 mL of the sample to a 50 mL centrifuge tube.
   c. Centrifuge the urine for 10 min at 5,000 × *g*.
   d. Discard the supernatant with a sterile serological pipette.
   e. Using a sterile filter tip, transfer 1000 μL of nuclease-free water to the sample pellet.
   f. Briefly vortex the tube to resuspend the pellet, then transfer the entire volume to a labelled 1.5 mL microfuge tube.
   g. Centrifuge the sample for 5 min at 10,000 × *g*.
   h. Using sterile filter tips, discard the supernatant.
   i. Freeze the urine pellet at −80°C until DNA extraction.

   Note: Although not necessary, nucleic acid preservative agents such as AssayAssure (Thermo Scientific) have been shown to aid urinary microbiome preservation (Jung et al., 2019). If such a preservative is to be used, it should be applied to all sample types in an equivalent manner to minimize confounding factors in the downstream sequencing analysis associated with processing conditions.

2. Ureteral stent sample preparation
   a. Ureteral stents should be collected during their routine removal by the treating urologist.
   b. Upon stent removal, the urologist should directly place the soiled device into a sterile urine collection container.
   c. Record the grade of encrustation of both proximal and distal ends of the stent (Table 2, Figure 1) then freeze the stent at −80°C until DNA extraction.

   Note: The proximal and distal ends of the device should be differentiated in order to appreciate any differences between the device-associated renal and bladder microbiota, respectively. The ends may be denoted by the urologist upon device removal, by the presence of marking indications from the manufacturer, or inferred by discoloration and encrustation differences (Kawahara et al., 2012, Chew et al., 2020, Chew and Denstedt, 2004).

Extraction of DNA from stent and urine samples

Timing: 3 days

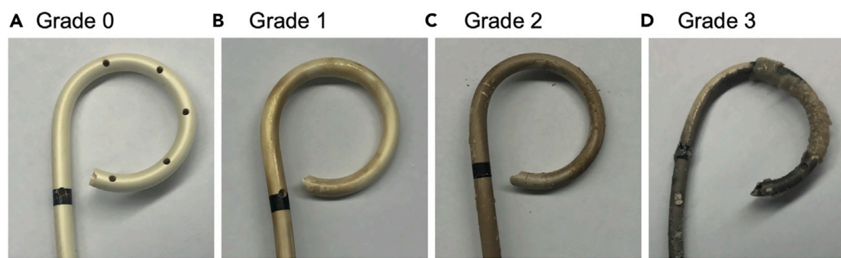


Figure 1. Examples of the classification of stent encrustation
(A) Grade 0, the stent appears like new with no visible fouling.
(B) Grade 1, the stent is discoloured.
(C) Grade 2, the stent harbours encrustation < 1 mm in thickness.
(D) The stent harbours encrustation > 1 mm in thickness.

This section describes the extraction of DNA from urine and stent samples.

3. Preparation of stent samples
   a. On the day of extraction, thaw the stent samples to 22°C and grade the degree of stent encrustation on both the proximal and distal ends of the stent. This should be done blinded to the outcome of the first encrustation grading prior to frozen storage.
   b. Inside the biosafety cabinet, use the razor blade sterilized with RNase AWAY to slice a 1 cm segment from each of the proximal and distal ends.
   c. To rinse the external surface of the stent segments, use forceps sterilized with RNase AWAY to hold one of the cut segments at a time over a sterile reservoir, and gently pipet 1 mL of nuclease-free water with a sterile filter-tip over the entire external surface of the segment.
   d. Sterilize the razor blade with RNase AWAY again, and slice open the rinsed segment lengthwise to expose the inner lumen.
   e. Add the sliced stent segment into the bead plate of the DNeasy PowerSoil HTP 96 Kit utilized for DNA extraction.

   Note: If both urine and stent samples are being investigated, they should be extracted together in the same plate with a completely randomized arrangement in order to minimize batch, plate, and edge effects in downstream analysis (Figure 2).

4. Preparation of urine samples
   a. Thaw the urine pellets at 22°C.
   b. Using a sterile filter tip, pipet 100 μL of nuclease free water onto one pellet, pipetting up and down until the sample becomes homogenous.

   Note: It is important to use water, not PBS, as it has been shown to be incompatible with the PowerSoil Kit (Hallmaier-Wacker et al., 2018).
   c. Transfer the sample into the bead plate of the DNeasy PowerSoil HTP 96 Kit utilized for DNA extraction.

5. Into one well of each plate add 100 μL of the nuclease free water used to pipet the urine pellet and wash the exterior of the stents. This will control for any contaminants in the water in the downstream analysis.
6. Leave two wells of the bead plate empty. One will act as a DNA extraction negative control, with no initial sample added but will undergo the entire extraction protocol; this controls for any contaminants in the extraction kit. The other well will act as a negative control for PCR amplification, with no DNA template being added to the PCR reaction; this controls for any contaminants in the PCR reagents (Figure 2).

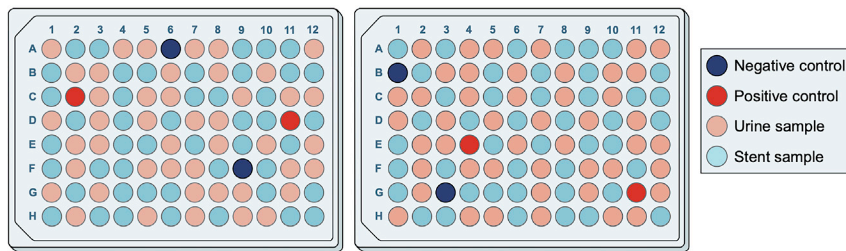


Figure 2. DNA extraction plate layout examples

Two plate layout examples are shown, but with the stated procedure, up to six plates may be sequenced at a time. The position of all clinical samples, positive, and negative controls should be randomized across the plate. Samples from the same patient should not be loaded in adjacent wells, and when comparisons are to be made between sample types, these should be distributed across different plates (for example, both urine and stent samples are mixed on each plate, as opposed to one plate for urine, another for stents).

7. Pipet the two positive controls with sterile filter tips into 2 wells of the bead plate.

   Note: As with the urine and stent samples, the position of the negative and positive controls should be randomized across plates.

8. Isolate DNA from the samples using the DNeasy PowerSoil HTP 96 Kit according to the manufacturer's instructions. All steps must be performed with sterile filter tips and are aided by the use of an electronic pipette.

   Note: To decrease the potential for contaminating aerosol generation, the electronic pipette's slowest motorized speed for aspirating and dispensing should be used.

   Note: Every time the plates are removed from the sterile biosafety cabinet to a non-sterile area such as the centrifuge, they must be wiped down with RNase AWAY prior to bringing them back into the sterile biosafety cabinet.

   a. Add 750 µL of PowerBead solution and 60 µL of Solution C1 to the bead plate loaded with all samples and controls. Seal the plates firmly with the square well mat. Label the positions A1 and H12 on the well mat with a permanent marker to avoid contamination from resealing the bead plate with the mat in the opposite position.

   Note: if Solution C1 has precipitated, heat to 60°C until it is fully dissolved.

   b. Shake plates for 20 min at speed 20 (20 oscillations per second) using the 96-well plate shaker, then centrifuge for 10 min (all centrifuge steps are conducted at 20°C at 2250 × *g*).
   c. Add 500 µL of the supernatant to a fresh plate with 250 µL Solution C2 and mix by pipetting up and down 3 times. Incubate plates at 4°C for 10 min followed by centrifugation for 10 min.
   d. Transfer the resulting supernatant to a fresh plate and repeat the centrifugation step. Transfer approximately 600 µL of the supernatant to a fresh plate containing 200 µL Solution C3 and mix by pipetting up and down 3 times.
   e. Incubate the plate at 4°C for 10 min followed by centrifugation for 10 min. Transfer the entire volume, with the exception of the pellet, to a fresh plate for centrifugation.

   Note: The pellet from Solution C3 can be extremely loose, so great care should be taken when transferring the supernatant.

   f. Carefully avoiding the loose residual pellet, transfer 650 µL from each well to a fresh plate containing 1300 µL Solution C4.
   g. Seal the plates with sealing tape.

   Pause point: At this point, the plates can be stored at 4°C for 12–24 h.

h. Briefly centrifuge the plates to avoid condensation transfer between wells after storing the plates for 12–24 h.
i. Remove sealing tape and mix the C4 sample solution by pipetting up and down. Transfer 500 μL of the solution to a Spin Plate, and centrifuge for 5 min.
j. Discard the flow-through and repeat i) until the entire sample-Solution C4 mix is processed through the Spin Plate.
k. Ensure the appropriate ethanol volume has been added to Solution C5-D, and add 500 μL of C5-D to the Spin Plate.
l. Centrifuge the Spin Plate twice for ten min, discarding the flow-through between spins.
m. Add 100 μL Solution C6 to the Spin Plate, incubate at 22°C in the biosafety hood for 10 min. Elute the DNA into the microplate by centrifuging for 15 min.
n. Measure the nucleic acid concentration using a Qubit fluorometer.

Note: Although DNA concentration may vary greatly by sample type and disease state (i.e., during a urinary tract infection), a minimum concentration that most samples should yield is 1 ng/μL.

o. Seal the microplate with the rubber strips provided in the kit using RNase AWAY-sterilized forceps, being careful not to touch the underside of the rubber stoppers. With a permanent marker, label the top of the rubber strips with A1-12, and the bottom of the strips with H1-H12.

Pause point: Store DNA at −20°C until downstream processing.

PCR amplification of the 16S rRNA gene V4 region library preparation

Timing: 4 h

This section describes PCR reaction set up and cycling conditions for sequencing the bacterial 16S rRNA gene V4 region with minimal contamination.

9. Array primers into 96-well microplates.
   a. Prepare primer stocks to 3.2 μM.
   b. Using a Biomek automated workstation in a UV-sterilized enclosure, dispense with filter tips 10 μL of the forward primers by row, and the reverse primers by column.

Note: For example, Plate 1 well H12 will contain Forward #4 and #Reverse 24. Plate 6 well H12 will contain Forward #24 and Reverse #24.

Note: If an automated liquid handling robot is not available, manual preparation may be performed in a UV-sterilized biosafety hood with PCR-grade filter tips and careful attention to pipetting precision.

10. If using DNA that has been stored frozen, thaw at 22°C and briefly centrifuge before removing the rubber strip caps inside the biosafety cabinet.
    a. To remove the rubber caps, use forceps sterilized with RNase AWAY and gently peel the strip off, being careful not to contact the underside of the strip.
    b. Place the strip on a UV-sterilized surface with one of 12 labels for each of columns 1–12.
    c. Seal the plate with a sealing mat to transfer to the sterilized Biomek enclosure, then unseal the plate.
11. Using the Biomek automated workstation with filter tips, transfer 2 μL of DNA (on average, this contains 20 ng of DNA) from the DNeasy PowerSoil microplate into the corresponding well of the primer plate.

Table 3. PCR cycling conditions

Steps	Temperature	Time	Cycles
Warm-up	95°C	4 min	1
Denaturation	95°C	1 min	25 cycles
Annealing	52°C	1 min	
Extension	72°C	1 min	
Hold	4°C	Forever	

Note: Other than the negative controls, template DNA concentration should be ≥ 1 ng/µL (Multinu et al., 2018). Any samples with concentrations >50 ng/µL can be diluted to 10 ng/µL.

12. Using a filter pipette tip, transfer 2000 µL of Promega GoTaq hot-start colorless master mix into a sterile reservoir inside the workstation.
13. Using the automated workstation and a multichannel 20 µL head, transfer 20 µL of the master mix from the reservoir to each well of the 96-well plate, with pipetting up and down to mix.
14. Seal the plates with foil plate covers very tightly and briefly centrifuge to remove air bubbles in the wells.
15. Carry out amplification in the thermocycler with the lid temperature maintained at 104°C, and the cycle conditions stated in Table 3.

16S rRNA amplicon sequencing procedure

Timing: 4 days

This section describes the process of sample clean-up, and the Illumina sequencing conditions. These methods were performed at London Regional Genomics Centre in London, Ontario, Canada (www.lrgc.ca).

16. Quantitate the concentration of the PCR amplicons using the PicoGreen dsDNA reagent kit and a fluorescent microplate reader (e.g., Molecular Devices SpectraMax M5) according to the manufacturer's instructions.

Note: To verify the size of the PCR amplicon (~290 bp), 1 µL of the PCR product may be run on a Bioanalyzer DNA chip.

17. Pool the amplicons equally such that approximately 50 ng of DNA per sample is loaded for sequencing.

Note: This amount of DNA may not be attainable from negative controls, but all samples should be loaded as equally as possible.

18. Perform the sequencing with an Illumina next-generation sequencer (e.g., MiSeq System) using a 600-cycle reagent kit. The sequencing run should be carried out as 2 × 260 cycles utilizing 5% Phi-X Control V3, with a cluster density of approximately 1100.

Note: When sequencing low bacterial abundance samples like urine and ureteral stents with the described methodology, as many as 500 samples may be run at a time and still achieve read depth capable of taxonomic profiling (>15 million reads per run). However, with specimens higher in diversity and bacterial abundance such as feces, higher per-sample read depth necessitates fewer samples per run. Exact read thresholds and sequencing depth will depend on the study question, environment being sampled, and sequencing technology.

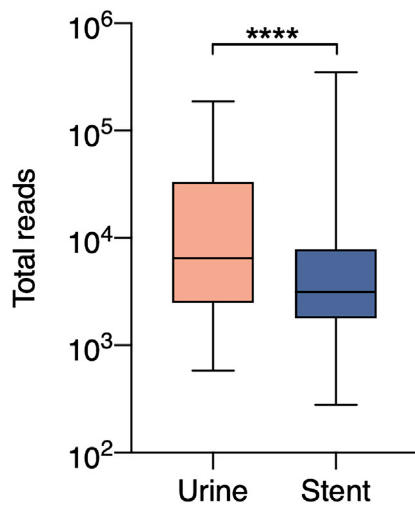


Figure 3. Post-filtering sample read count
The total read count was higher in urine samples compared to stents (Mann-Whitney U test, $P < 0.0001$). Box plot whiskers represent minimum and maximum. Graph generated with GraphPad Prism v8.3.1. Figure reprinted with permission from Al et al., 2020.

EXPECTED OUTCOMES

Accurate microbiota analysis of low-bacterial-abundance samples requires a processing methodology that maximizes DNA yield and minimizes contamination. This protocol presents a reliable method of sample processing to assess the microbiota from human urine and ureteral stent samples, even those that would historically be referred to as "sterile" (Whiteside et al., 2015). Similarly, it is important to apply appropriate downstream quality filtering in the analysis of such samples.

A successful DNA extraction and 16S rRNA gene (V4) PCR amplification yields dsDNA amplicon concentrations ranging from 5–40 ng/μL for urine and stent samples, ≤3 ng/μL for the negative and 50 ng/μL for the positive controls. When sequenced under these conditions and after the downstream quality filtering described below, samples will yield average read depths of ~30,000 and ~20,000 for urine and stents, respectively (Figure 3).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Upon completion of the sequencing run, perform a quality check on the files with FastQC (Andrews, 2010).
2. Demultiplex the reads and generate a sequence variant (SV) count tables using the DADA2 pipeline and the most recent SILVA taxonomic training set (Callahan et al., 2016, Quast et al., 2013).

   Note: SVs are high-resolution analogs of traditional operational taxonomic units (OTUs) which provide improved accuracy in terms of taxonomic classification and are directly comparable across different studies.

3. For studies comprising more than one sequencing run, SV counts from each run must be merged together.
   a. Several samples as well as negative and positive controls should be sequenced in multiplicate (on every run), then can be compared downstream using principal component analysis (for example with the R package CoDaSeq) to determine the presence of sequencing batch effects (Gloor and Reid, 2016, Team, 2019).
   b. If no batch effects dominate the sequences, SVs that are not consistently detected in all runs should be removed, and the remaining counts then merged.
   c. Prune the merged SV table such that the final dataset utilized in all downstream analyses retains samples with greater than 1,000 filtered reads, SVs present at 1% relative abundance

Table 4. Metadata features relevant to urinary microbiota studies

Category	Metric
Sample processing details	Date of sample collection and processing
	Total urine sample volume from which pellet was derived
	Processing time (were some samples processed immediately while others after several h at 22°C)
	Sample position on DNA extraction or PCR plate
	Pre- and Post- PCR DNA concentration
Patient factors	Age
	Sex
	Weight
	BMI
	Urinalysis and blood test results
	Clinical urine culture results
	Current comorbidities
	Previous medical history
	Smoking status
	Diet (assessed through a validated diet history questionnaire or food diary)
	Medication use (including both prescription and over the counter)
	Supplement use (vitamins, herbal remedies, etc.)
	Previous antibiotic use
	Physical activity level

or greater in any sample, and SVs with greater than 1,000 total reads across all samples in all runs. Any SVs corresponding to human mitochondrial sequences should also be removed.

Note: These filtering criteria will not be applicable to all studies, and will depend on the study question, environment being sampled, and sequencing technology.

4. Additional filtering should remove taxa common in the negative controls, indicative of contaminants that may be present in the water, DNA extraction kit, PCR reagents, or other sources (Glassing et al., 2016). The computational R package 'decontam' is effective in determining contaminating sequences based on their prevalence and frequency in negative controls (Davis et al., 2018).
5. Downstream analysis should be undertaken with a compositional approach, as has been described previously (Gloor et al., 2017). Appropriate tools developed for these analyses include ALDEx2, CoDaSeq, MaAsLin2, and vegan, among many other R packages (Gloor and Reid, 2016, Fernandes et al., 2013, Mallick et al., 2020, Oksanen et al., 2019). A thorough analysis of microbiota data should include distance-based metrics (such as Aitchison's distance), diversity metrics (for example, Shannon's Index of alpha diversity), identification of confounders, and where relevant, multivariate analysis. See Al et al., 2020 for examples of all of these analyses with urine and stent samples. Relevant metadata features that should be considered in study design and data collection are described in Table 4.
6. Correctly upload all data to a public repository such as the NCBI SRA or EBI. This should include sequence barcode information, methodological and processing details, and de-identified clinical metadata.

LIMITATIONS

This protocol was developed to provide an accurate assessment of the low-biomass bacterial community within human urine and ureteral stent samples in the absence of infection. While this method procures accurate, reliable, and repeatable results, there are several limitations that investigators should be aware of.

In some extremely low bacterial abundance samples (approximately 10% of total samples on average), the described protocol is unable to generate sequencing libraries that surpass post-sequencing quality filtering cut-offs or differ substantially from negative control samples. In these cases, samples should be removed from downstream analysis, and thus a 10% contingency should be accounted for during experimental design and power calculations. Removing these samples from downstream analysis inherently biases the findings towards those with higher bacterial abundance, which may lead to significant artifacts in the results depending on the study design. An alternate, ultra-sensitive method of DNA extraction may be considered for crucial samples, or an entirely different methodological approach may be warranted instead of 16S rRNA amplicon sequencing (Costantini et al., 2018).

The described protocol relies on a large sample volume (> 20 mL) of clean-catch midstream urine, which may not be suitable for every clinical study, including those that collect intra-operative specimens or samples from children. For intra-operative sample collection, transurethral catheter or suprapubic aspirate may be considered as alternative means (Wolfe et al., 2012).

The surface of the stent is in contact with the urethra, periurethral area, and instrumentation of the physician during stent removal, then is stored with proximal and distal curls in contact with each other, so it is important that the exterior of the stent segment is gently rinsed to remove surface contaminants prior to DNA extraction. However, by rinsing, the protocol as described reduces information gathered from the outside of the stent, which may include the presence of clinically relevant bacteria.

Characterization of urinary tract-derived bacterial communities via 16S rRNA gene-based profiling, as is outlined here, is unable to differentiate between uropathogenic strains of *E. coli* (or other relevant uropathogens). Accordingly, while 16S rRNA gene sequencing methods can be informative and provide useful complementary analysis in cases of subclinical infection, investigators should not attempt to use these methods in replacement of standard diagnostic microbiology.

The described protocol is unable to detect fungal species found at ultra-low abundance within the urinary "mycobiome" (Ackerman and Underhill, 2017). Though, this is an intrinsic shortcoming of bacterial 16S rRNA gene sequencing rather than the described DNA extraction protocol itself, which yields high quality genomic DNA from bacteria, fungi, and protozoa. Thus, established methods such as ITS or 18S rRNA gene sequencing can be applied to the extracted DNA samples (following Step 7) in cases of urological chronic pelvic pain syndrome, post-kidney transplant urinary tract infection, or other instances where fungal species may be of particular interest (Nickel et al., 2020, Li et al., 2020).

TROUBLESHOOTING

Problem 1

The urine pellet is larger than what could be pipetted in 100 μL of water. (step 4.b)

Potential solution

Due to the presence of urinary crystals, blood, infection, and other substances, the pellet may be too large to distribute and pipette in 100 μL of water. However, it is crucial that the entire pellet should be utilized wherever possible. Importantly, pellet size increases from urinary salt precipitation following refrigeration, which in turn can decrease sample DNA yield, so ensure that samples are not refrigerated prior to processing (Ackerman et al., 2019). To transfer a very large pellet to the bead plate it may be necessary to pipette quite slowly using wide-bore tips. Additional water may also be added to distribute the pellet, up to a total volume in the bead plate of 250 μL.

Problem 2

Low DNA yield. (step 8.n)

Potential solution

The DNA extraction process with the PowerSoil kit may be modified to include a heated lysis step to optimize the final yield. After the addition of PowerBead solution and Solution C1 (step 7a), seal the plates with the square well mat and place them in a 65°C dry bath for 10 min prior to shaking (step 7b). Whether this step is necessary should be validated by troubleshooting (for example by PCR of the 16S rRNA gene followed by visual confirmation of successful amplification by routine gel electrophoresis and DNA staining methods) prior to commencing a microbiota study, where all plates must be processed identically.

Additionally, the PowerSoil kit has been documented to be incompatible with samples in PBS storage (Hallmaier-Wacker et al., 2018). To optimize the kit yield and downstream output, ensure that prior to DNA extraction, urine pellets and stents are not stored in PBS.

Problem 3

Following PCR amplification, the well volume is inconsistent. (step 15)

Potential solution

The foil plate seal must be very tightly applied, with specific attention to detail on the edge and corner wells of the 96-well plate. If evaporation still occurs, the lid of the thermocycler may have inconsistent heating to 104°C, and an alternate thermocycler should be used. Finally, if the lid of the thermocycler is not in even contact with the 96-well plate, an alternate 96-well plate brand may be used, or aluminum foil cut to size of the 96-well plate lid can be placed on top of the plate to fill the gap.

Problem 4

A low percentage of reads are mapped. (step 2)

Potential solution

Ensure that the most recent and appropriate taxonomic training set has been used for mapping (Quast et al., 2013). Poor sequence quality may also be responsible for low mapping and should be assessed with FastQC (Andrews, 2010). Finally, the percentage of PhiX spike-in control used during the Illumina sequencing may play a role in mapping rates. Low bacterial abundance samples are particularly vulnerable to overshadowing from the misloading of PhiX, so ensure that only 5% was utilized in the sequencing run. If this was not the case, the sequencing may need to be repeated.

Problem 5

Samples have very low or zero total number of reads. (step 2)

Potential solution

Assuming the PCR amplicon concentration was suitable for sequencing, there may be an error with demultiplexing. Ensure unique and suitable sample names are used and confirm the appropriate assignment of barcode indices to each sample.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jeremy P. Burton (Jeremy.Burton@lawsonresearch.com).

Materials availability

This study did not generate any unique materials or reagents.

Data and code availability

This study did not generate any unique datasets or code, but 16S rRNA gene sequencing data that this study is associated with is available through the NCBI Sequence Read Archive, BioProject ID #PRJNA601180.

ACKNOWLEDGMENTS

This study was funded by the W. Garfield Weston Foundation. The authors would like to thank David Carter at the London Regional Genomics Centre (www.lrgc.ca) for facilities and resources for the study.

AUTHOR CONTRIBUTIONS

J.B. conceived the project, acquired funding, and supervised the work. K.A. performed the experiments and data analysis. K.A. and J.B. wrote and reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Ackerman, A.L., Anger, J.T., Khalique, M.U., Ackerman, J.E., Tang, J., Kim, J., Underhill, D.M., Freeman, M.R., and NIH Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP). (2019). Optimization of DNA extraction from human urinary samples for mycobiome community profiling. PLoS One *14*, e0210306.

Ackerman, A.L., and Underhill, D.M. (2017). The mycobiome of the human urinary tract: potential roles for fungi in urology. Ann. Transl. Med. *5*, 31.

Al, K.F., Denstedt, J.D., Daisley, B.A., Bjazevic, J., Welk, B.K., Pautler, S.E., Gloor, G.B., Reid, G., Razvi, H., and Burton, J.P. (2020). Ureteral stent microbiota is associated with patient comorbidities but not antibiotic exposure. Cell Rep. Med. *1*, 100094.

Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bundgaard-Nielsen, C., Ammitzboll, N., Isse, Y.A., Muqtar, A., Jensen, A.M., Leutscher, P.D.C., Arenholt, L.T.S., Hagstrom, S., and Sorensen, S. (2020). Voided urinary microbiota is stable over time but impacted by post void storage. Front. Cell. Infect. Microbiol. *10*, 435.

Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., and Holmes, S.P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods *13*, 581–583.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. *6*, 1621–1624.

Chew, B.H., Chan, J., Choy, D., Hirayama, T., Iwamura, M., Branda, N., and Lange, D. (2020). The interaction of urinary components with biomaterials in the urinary tract: ureteral stent discoloration. J. Endourol. *34*, 608–616.

Chew, B.H., and Denstedt, J.D. (2004). Technology insight: novel ureteral stent materials and designs. Nat. Clin. Pract. Urol. *1*, 44–48.

Costantini, L., Magno, S., Albanese, D., Donati, C., Molinari, R., Filippone, A., Masetti, R., and Merendino, N. (2018). Characterization of human breast tissue microbiota from core needle biopsies through the analysis of multi hypervariable 16S-rRNA gene regions. Sci. Rep. *8*, 16893.

Davis, N.M., Proctor, D.M., Holmes, S.P., Relman, D.A., and Callahan, B.J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome *6*, 226.

Fernandes, A.D., Macklaim, J.M., Linn, T.G., Reid, G., and Gloor, G.B. (2013). ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-Seq. PLoS One *8*, e67019.

Glassing, A., Dowd, S.E., Galandiuk, S., Davis, B., and Chiodini, R.J. (2016). Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples. Gut Pathog. *8*, 24.

Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., and Egozcue, J.J. (2017). Microbiome datasets are compositional: and this is not optional. Front. Microbiol. *8*, 2224.

Gloor, G.B., and Reid, G. (2016). Compositional analysis: a valid approach to analyze microbiome high-throughput sequencing data. Can. J. Microbiol. *62*, 692–703.

Hallmaier-Wacker, L.K., Lueert, S., Roos, C., and Knauf, S. (2018). The impact of storage buffer, DNA extraction method, and polymerase on microbial analysis. Sci. Rep. *8*, 6292.

Jung, C.E., Chopyk, J., Shin, J.H., Lukacz, E.S., Brubaker, L., Schwanemann, L.K., Knight, R., Wolfe, A.J., and Pride, D.T. (2019). Benchmarking urine storage and collection conditions for evaluating the female urinary microbiome. Sci. Rep. *9*, 13409.

Karstens, L., Asquith, M., Caruso, V., Rosenbaum, J.T., Fair, D.A., Braun, J., Gregory, W.T., Nardos, R., and Mcweeney, S.K. (2018). Community profiling of the urinary microbiota: considerations for low-biomass samples. Nat. Rev. Urol. *15*, 735–749.

Kawahara, T., Ito, H., Terao, H., Yoshida, M., and Matsuzaki, J. (2012). Ureteral stent encrustation, incrustation, and coloring: morbidity related to indwelling times. J. Endourol. *26*, 178–182.

Li, T., Huang, Y., Chen, X., Wang, Z., and Xu, Y. (2020). Urinary tract infections caused by fluconazole-resistant trichosporon japonicum in 2 kidney transplant patients and analysis of their homology. Open Forum Infect. Dis. *7*, ofaa365.

Mallick, H., Rahnavard, A., and Mciver, L. (2020). Maaslin2: maaslin2 [online]. http://huttenhower.sph.harvard.edu/maaslin2.

Multinu, F., Harrington, S.C., Chen, J., Jeraldo, P.R., Johnson, S., Chia, N., and Walther-Antonio, M.R. (2018). Systematic bias introduced by genomic DNA template dilution in 16S rRNA gene-targeted microbiota profiling in human stool homogenates. mSphere *3*, e00560-17.

Nickel, J.C., Stephens, A., Landis, J.R., Mullins, C., Van Bokhoven, A., Anger, J.T., Ackerman, A.L., Kim, J., Sutcliffe, S., Krol, J.E., et al. (2020). Urinary fungi associated with urinary symptom severity among women with interstitial cystitis/bladder pain syndrome (IC/BPS). World J. Urol. *38*, 433–446.

Oksanen, J.F., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R., O'hara, R.B., Simpson, G.L., Solymos, P., et al. (2019). vegan: community ecology package. R package version 2.5-6 [Online]. https://CRAN.R-project.org/package=vegan.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glockner, F.O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. *41*, D590–D596.

Ribeiro, K.C.B., Serabion, B.R.L., Nolasco, E.L., Vanelli, C.P., Mesquita, H.L.D., and Corrêa,

J.O.D.A. (2013). Urine storage under refrigeration preserves the sample in chemical, cellularity and bacteriuria analysis of ACS. J. Bras. Patol. Med. Lab. *49*, 415–422.

Southworth, E., Hochstedler, B., Price, T.K., Joyce, C., Wolfe, A.J., and Mueller, E.R. (2019). A cross-sectional pilot cohort study comparing standard urine collection to the peezy midstream device for research studies involving women. Female Pelvic Med. Reconstr. Surg. *25*, e28–e33.

Team, R.C. (2019). R: A language and environment for statistical computing (R Foundation for Statistical Computing).

Whiteside, S.A., Razvi, H., Dave, S., Reid, G., and Burton, J.P. (2015). The microbiome of the urinary tract–a role beyond infection. Nat. Rev. Urol. *12*, 81–90.

Wolfe, A.J., Toh, E., Shibata, N., Rong, R., Kenton, K., Fitzgerald, M., Mueller, E.R., Schreckenberger, P., Dong, Q., Nelson, D.E., and Brubaker, L. (2012). Evidence of uncultivated bacteria in the adult female bladder. J. Clin. Microbiol. *50*, 1376–1383.