FOOD SAFETY LABORATORY
CORNELL UNIVERSITY

Name of Protocol

FILE NAME: High-Throughput gDNA Extraction (Qiagen)

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to provide detailed instructions on isolating high quality genomic DNA from bacteria for Illumina Nextera XT preps and subsequent full genome sequencing. However, this protocol can be used for other applications where high quality genomic DNA is required.

1.2 Scope

This SOP applies to the Food Safety Lab and Milk Quality Improvement Program

1.3 Definitions

Lysozyme – Enzyme that attacks and damages peptidoglycans in bacterial cell walls. Used to improve lysis of gram-positive bacteria.

RNase – Nuclease that catalyzes the degradation of RNA into its respective subunits.

Proteinase K – Broad-spectrum serine protease. Used to inactivate and destroy DNases and RNases in cell lysates.

A260/A280 ratio – "Used as a measure of purity in both nucleic acid and protein extractions. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA."

A260/A230 ratio – "A secondary measure of nucleic acid purity in DNA/RNA extractions. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm."

1.4 Safety

If isolating DNA from BSL-2 organisms (e.g. *Listeria, Salmonella, etc.*), appropriate protective measures need to be taken. All BSL-2 waste needs to be treated accordingly.

SECTION 2 MATERIALS

Gram Positive

- Lysozyme
- Stock EDTA
- Triton X-100

Gram Positive and Negative

- Stock Tris·HCl (pH 8.0)
- RNase A (100 mg/mL; cat. no. 19101)
- Ethanol (96-100%)
- Sterile inoculating toothpicks
- Eppendorf 5810R Centrifuge with deep-well buckets
- DNeasy 96 Blood & Tissue Kit
- 1.3 or 2 mL DeepWell Plates
- Reagent reservoirs for multichannel pipetting
- Multichannel pipets
- Costar 3370 clear 96-well plates
- 96-well plate foil seals (optically clear plastic seals are an alternative)
- Biosafety Cabinet (optional)

If growing cultures on agar plates:

- Phosphate Buffered Saline (pH 7.4)
- Sterile inoculating loop or swab

SECTION 3 PROCEDURES

Note: The protocol shown below is taken directly from the QIAamp DNA Mini & Blood Mini Handbook with modifications made where necessary. For example, adaptations from the DNeasy 96 Blood & Tissue Kit Handbook are incorporated into the protocol where necessary. All modifications are in italics. Only applicable steps and excerpts from the handbook are shown. The full version is available through www.qiagen.com

3.1 Tips before starting

- *If harvesting cells from plate cultures, do not use too many cells. Too large of a pellet can result in incomplete lysis and clogging of the column in subsequent steps.* Maximum cells should be 2 x 10^9.
- *Be gentle when vortexing and do not vortex more than instructed. You do not want to over-shear the DNA.*
- *Using the provided S-block in our Eppendorf 5810R centrifuge restricts movement of the buckets and consequently prevents our centrifuge from functioning properly. Therefore, we use the supplied elution racks to pass filtrate into during column spin steps. Costar 3370 plates (clear) serve as our elution racks.*
- *Make sure all centrifugations are balanced. Executing this procedure with one DNeasy 96 plate will require a balance throughout the protocol.*
- *Do not add ethanol to Buffer AL despite the instructions on the bottle.*

3.2 Isolation of genomic DNA from Gram-positive and Gram-negative bacteria

1. *In a BioSafety Cabinet or working in sterile conditions under a flame, inoculate one or two 96-DeepWell plates containing 1.0 mL sterile nutrient media. Seal DeepWell plate with an adhesive lid, either foil or plastic, and incubate overnight according to the bacteria's growth requirements.*

   If bacteria do not grow well in liquid culture:

   i. *Isolate bacteria from fresh culture plate using an inoculation loop/sterile swab*

   ii. *Homogenize bacteria in 1.0 mL PBS within the 96-DeepWell plate (gram-positive) or in 180* µl *Buffer ATL within the Qiagen collection microtubes (gram-negative). If working with gram-positives from plates, seal DeepWell plate and proceed to step 2 below. If working with gram-negatives from plates,* proceed to step 3 in section 3.3: "DNA Purification from Tissues".
2. Pellet bacteria in *96-DeepWell plate(s)* by centrifugation at *full speed (4,000 rpm) for 15 min. Use the Eppendorf 5810R centrifuge.* Remove all supernatant without disturbing pellet. *This can be done quickly using a vacuum aspiration system (tubing can be connected to a non-filter p1000 tip which has a non-filter p200 tip fitted on the end). Make sure to change p200 tips between each well while aspirating.*

3. Using a multichannel pipette, suspend bacterial pellet in 180 µl of the appropriate enzyme solution (20 mg/ml lysozyme or 200 µg/ml lysostaphin; 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton) *or Buffer ATL*. *Use lysozyme mixture for gram-positives and Buffer ATL for gram-negatives. Pipet 6x to homogenize the bacterial suspension. If working with gram-negatives, p*roceed to step 3 in section 3.3: "DNA Purification from Tissues". Note: *FSL and MQIP use lysozyme, not lysostaphin.*
4. *Seal DeepWell plate(s) with foil or plastic cover and incubate in water bath for 30-75 min at 37°C (see references). Gently disturb DeepWell plate(s) to mix solution after 15 min of incubation.*
5. *Transfer bacteria-lysis suspension to Qiagen collection microtubes.*
6. Add 20 µl proteinase K (from kit), *seal with microtube caps, and shake collection microtubes by inversion while keeping one hand over microtube caps. Centrifuge briefly to collect liquid. If RNA-free genomic DNA is required, incubate in water bath at 56°C for 10 min and proceed to step 7. If RNA-free genomic DNA is not required, incubate in water bath at 56°C for 45 min. and proceed to step 8.*
7. *Let liquid cool to room temperature. Add 4µl RNase (100mg/ml). Seal collection microtube with new microtube caps and gently disturb/rock microtube rack to mix. Incubate at room temperature for 10 min.*
8. Using a *multichannel pipette*, add 200 µl Buffer AL, add new microtube caps, and mix by *shaking thoroughly while keeping one hand over microtube caps*. *Centrifuge briefly to collect liquid. If RNase treatment was performed, complete the proteolytic digestion with an additional incubation at 56℃ for 30 min.*
9. Incubate at 70°C in a water bath for 10 min to inactivate any remaining enzymes. *Cool to room temperature and centrifuge for a few seconds to collect liquid.*
10. Follow the "Protocol: DNA Purification from Tissues" from step 6 under section 3.3.

3.3 DNA Purification from Tissues (QIAamp DNA Mini Kit Protocol Adapted for DNeasy 96 Kit)

3. Add 20 µl proteinase K, and *thoroughly shake collection microtubes by inversion while keeping one hand over microtube caps.* Incubate in water bath at 56°C until the tissue is completely lysed (*45-60 min*). *Gently disturb/rock microtube rack* occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. To ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, mixing 2–3 times per hour during incubation is recommended.

4. Briefly centrifuge the *collection microtube rack* to remove drops from the inside of the microtube caps.

5. If RNA-free genomic DNA is required, follow step 5a. Otherwise, follow step 5b.
RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.

5a. First add 4 µl RNase A (100 mg/ml), *seal collection microtube with new microtube caps and gently disturb/rock microtube rack to mix.* Incubate for 10min at room temperature (15–25°C). *Using a multichannel pipet,* add 200 µl Buffer AL to the sample. *Mix by shaking thoroughly while keeping one hand over microtube caps. Briefly centrifuge the collection microtube* to remove drops from inside the lid, and incubate at 70°C for 10 min.
It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5b. *Using a multichannel pipet,* add 200 µl Buffer AL to the sample. *Add new microtube caps and mix by shaking thoroughly while keeping one hand over microtube caps. Briefly centrifuge the collection microtube* to remove drops from inside the lid, and incubate at 70°C for 10 min.
It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

6. *Using a multichannel pipet,* add 200 µl ethanol (96–100%) to the sample*, add new microtube caps, and mix by shaking collection microtubes thoroughly while keeping one hand over strip caps*. After mixing, briefly centrifuge the *microtube rack* to remove drops from inside the lid.
It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the *DNeasy column (we do not transfer everything in this procedure)*. This precipitate does not interfere with the *DNeasy* procedure or with any subsequent application. Do not use alcohols other than ethanol since this may result in reduced yields.

7. Carefully apply *550 uL* of the mixture from step 6 (including the precipitate) to the *DNeasy column(s) (in an old elution microtube rack)* without wetting the rim. *This will have to be completed in three transfers using the p200 multichannel. Seal the DNeasy column(s) with the provided AirPore tape sheets and centrifuge at 4,000 rpm for 10 minutes. Discard filtrate and rinse elution microtube rack. Place the DNeasy column(s) back in the rinsed elution microtube rack.*
* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 of *QIAamp DNA Mini & Blood Mini* handbook for safety information.

8. *Remove AirPore tape sheet from DNeasy column(s)* and add 500 µl Buffer AW1 <u>without wetting the rim.</u> *This will have to be completed in three transfers using the p200 multichannel. Seal the DNeasy column(s) with a new AirPore tape sheet and centrifuge at 4,000 rpm for 5 minutes. Discard filtrate and rinse elution microtube rack. Place the DNeasy column(s) back in the rinsed elution microtube rack.*

9. *Remove AirPore tape sheet from DNeasy column(s)* and add 500 µl Buffer AW2 without wetting the rim. *This will have to be completed in three transfers using the p200 multichannel.* Seal the *DNeasy column(s) with a new AirPore tape sheet* and centrifuge at *4,000 rpm for 10 minutes*.

10. Recommended: Place the *DNeasy column(s)* in a new *elution microtube rack*. *Discard filtrate and rinse the old elution microtube rack. Seal the DNeasy column(s) with a new AirPore tape sheet and centrifuge at 4,000 rpm for 5 min*.
This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. *Pre*-warm *Tris-HCL (to be used for elution) at 37°C.* Place the DNeasy column(s) in a *clean Costar 3370 clear 96-well plate (not provided)*. *Remove AirPore tape sheet from DNeasy column* and add *50*µl Tris-*HCL* directly to the column *using a multichannel pipet.* Incubate at room temperature for *4 min*, and then centrifuge at *4,000 rpm* for 3 min. *Rinse the elution microtube rack used for the dry spin*.

12. Repeat step 11 using new *Costar 3370 clear 96-well plate labeled elution 2*.
A third elution step with a further 200 µl *Tris-HCL* AE will increase yields by up to 15%.

Volumes of more than 100 µl should not be eluted into a *Costar 3370 96-well plate* because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation. Lower elution volumes increase the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 25 in *QIAamp DNA Mini & Blood Mini Handbook*). Eluting with 4 x 50 µl instead of 2 x 100 µl does not increase elution efficiency.

****For long-term storage of DNA, eluting in *Tris-HCL* and placing at –20°C is recommended**, since DNA stored in water is subject to acid hydrolysis. Yields of DNA will depend both on the amount and the type of tissue processed. 25 mg of tissue will yield approximately 10–30 µg of DNA in 400 µl of water (25–75 ng/µl), with an A260/A280 ratio of 1.7–1.9. For more information about elution and how to determine DNA yield, length, and purity, refer to pages 24–25 and Appendix A, page 50 of the QIAamp DNA Mini & Blood Mini Handbook.

13. Quantify DNA using the Take 3 adapter on the Synergy H1 Plate Reader. The Take 3 adapter allows up to 16 samples to be quantified simultaneously. See protocol "8.3.15_Take3_NucleicAcid_Quantification" on Food Safety Wiki.

SECTION 4 TROUBLESHOOTING

- Elution: Water, Tris-HCL, or Tris-EDTA (Buffer AE) can be used for eluting DNA. Water pH can vary which leaves the DNA susceptible to acid hydrolysis during freeze-thaw cycles. Tris-HCL and Tris-EDTA are prepared to an optimal pH for DNA storage. EDTA, a chelator, can buffer primer degradation if contaminant nucleases are present. However, EDTA has the potential to inhibit downstream enzymatic processes. Due to these reasons, Tris-HCL is an ideal eluting option, especially if long term storage is required.
- When quantifying DNA, make sure to gently mix and warm up samples if they have sat in the cold room. Genomic DNA can precipitate out of solution.
- Low A260/A280 ratios may be caused by: Carryover of protein, salt, and other reagents associated with the extraction protocol and/or very low concentration (> 10 ng/ul) of nucleic acid.
- Low A260/230 ratios may be caused by: Carryover of salt, carbohydrates, peptides, phenol, TRIzol, guanidine HCL (DNA isolations), guanidine isothiocyanate (RNA isolations), or aromatic compounds in general.
- DNA from the 2nd elution has been shown to have a higher molecular weight. (See Steven Warchocki "DNA Prep For Full Genome" notebook page 16)

SECTION 5 REFERENCES

QIAamp DNA Mini & Blood Mini Handbook

DNeasy Blood & Tissue Handbook

http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf

http://www.bio.davidson.edu/projects/gcat/protocols/NanoDrop_tip.pdf

http://www.qiagen.com/knowledge-and-support/faq/?ID=c59936fb-4f1e-4191-9c16-ff083cb24574

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC252029/pdf/jbacter00400-0140.pdf