

Journal of Visualized Experiments

Quasimetagenomic analysis of Salmonella from food and environmental samples

--Manuscript Draft--

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| Article Type: | Invited Methods Article - JoVE Produced Video |
| Manuscript Number: | JoVE58612R2 |
| Full Title: | Quasimetagenomic analysis of Salmonella from food and environmental samples |
| Keywords: | quasimetagenomics, immunomagnetic separation (IMS), whole genome amplification, , sequencing, detection, subtyping, Salmonella |
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| Additional Information: | |
| Question | Response |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US\$2,400) |
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Dear Editor,

Please find enclosed our manuscript titled "Sample preparation for quasimetagenomic analysis of Salmonella from food and environmental samples"

We previously described a quasimetagenomics approach that combines Salmonella detection and subtyping in a single workflow. That work was published in 2018 February issue of Applied and Environmental Microbiology (AEM) and featured by AEM spotlight. We believe that a visualized and detailed protocol with an expanded list of representative samples will facilitate the use of this approach by researchers and food safety professionals, especially potential users from the food industry that prefer an easy-to-follow protocol.

Author contribution:

XD designed the study. JH, DM and AT performed the experiment. JH, DM and XD wrote the paper.

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I look forward to hearing from you.

Sincerely,

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TITLE:

Quasi-Metagenomic Analysis of *Salmonella* from Food and Environmental Samples

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KEYWORDS:

quasimetagenomics, immunomagnetic separation (IMS), whole genome amplification, sequencing, detection, subtyping, *Salmonella*

SHORT ABSTRACT:

Here, we present a protocol to prepare DNA samples from food and environmental microbiomes for concerted detection and subtyping of *Salmonella* through quasimetagenomic sequencing. The combined use of culture enrichment, immunomagnetic separation (IMS), and multiple displacement amplification (MDA) allows effective concentration of *Salmonella* genomic DNA from food and environmental samples.

LONG ABSTRACT:

Quasi-metagenomics sequencing refers to the sequencing-based analysis of modified microbiomes of food and environmental samples. In this protocol, microbiome modification is designed to concentrate genomic DNA of a target foodborne pathogen contaminant to facilitate the detection and subtyping of the pathogen in a single workflow. Here, we explain and demonstrate the sample preparation steps for the quasi-metagenomics analysis of *Salmonella enterica* from representative food and environmental samples including alfalfa sprouts, ground black pepper, ground beef, chicken breast and environmental swabs. Samples are first subjected to the culture enrichment of *Salmonella* for a shortened and adjustable duration (4-24 h). *Salmonella* cells are then selectively captured from the enrichment culture by immunomagnetic separation (IMS). Finally, multiple displacement amplification (MDA) is performed to amplify DNA from IMS-captured cells. The DNA output of this protocol can be sequenced by high throughput sequencing platforms. An optional quantitative PCR analysis can be performed to replace sequencing for *Salmonella* detection or assess the concentration of *Salmonella* DNA before sequencing.

INTRODUCTION:

Metagenomics sequencing theoretically allows concerted detection and subtyping of foodborne pathogens. However, food samples present challenges to the pathogen analysis by direct sequencing of the food microbiome. First, foodborne pathogens are often present at low levels in food samples. Most of the commercially available rapid detection methods still require 8 - 48 h culturing to enrich pathogen cells to a detectable level¹. Second, many foods contain abundant microflora cells and/or food DNA, making foodborne pathogen DNA a small fraction of food metagenome and an elusive target for detection and subtyping by direct metagenomic sequencing.

Modification of food microbiomes has been reported to allow substantial concentration of foodborne pathogen DNA to facilitate sequencing-based detection of Shiga toxin-producing *Escherichia coli*^{2,3} and *Salmonella enterica*⁴. Because modified food microbiomes are still mixtures of different microbial species, their sequencing is termed as quasimetagenomic analysis⁴. Microbiome modification can be performed by culture enrichment alone^{2,3} or in combination with immunomagnetic separation (IMS) and multiple displacement amplification (MDA)^{4,5}. IMS can selectively capture pathogen cells from the enrichment culture via antibody-coated magnetic beads. MDA can generate sufficient amounts of genomic DNA for sequencing through the highly efficient ϕ 29 DNA polymerase⁶. IMS-MDA has allowed culture-independent pathogen detection from clinical samples⁷, and shortening of the culture enrichment for quasimetagenomic detection and subtyping of *Salmonella* in food samples⁴.

The overall goal of this method is to prepare quasi-metagenomic DNA from food samples to allow targeted concentration of *Salmonella* genomic DNA and subsequent detection and subtyping of the *Salmonella* contaminant by sequencing. Compared to standard methods for *Salmonella* detection^{8,9} and subtyping¹⁰, the quasimetagenomic approach can substantially shorten the turnaround time from contaminated food and environmental samples to molecular subtypes of the pathogen by unifying the two typically separated analyses into a single workflow. This method is particularly useful for applications such as foodborne outbreak response and other trace back investigations where robust pathogen subtyping is required in addition to pathogen detection and rapid analytical turnaround is important.

PROTOCOL:

1. Sample Preparation

Note: Food samples are prepared for pre-enrichment according to Microbiology Laboratory Guidebook (MLG) of U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS)¹¹ and Bacteriological analytical manual (BAM) of U.S. Food and Drug Administration (FDA)¹².

1.1. Aseptically place a 25 g portion of food sample such as black pepper, chicken breast, ground beef, and alfalfa sprouts or an environmental swab into a sterile laboratory blender bag with a built-in filter. Prepare an environmental swab by aseptically moistening a sponge with enrichment broth (Rappaport-Vassiliadis, RV). Then, drag the swab over entire surface or predetermined area and place it into a laboratory blender bag.

[place **Figure 1** here].

1.2. Thoroughly mix each 25 g sample with 225 mL of RV enrichment broth using a laboratory blender or hand massage for 30 s.

Note: Variants of RV broth as recommended by MLG and BAM may be used depending on sample types.

1.3. Incubate sample-enrichment broth mixture in a laboratory incubator at 42 °C for 4 – 24 h.

1.4. After incubation, collect a 50 mL subsample of enrichment broth from the filtered side of the bag in a separate 50 mL centrifuge tube.

1.5. Centrifuge the tube at 100 × g for 10 min to remove solid debris in the homogenate.

1.6. Recover the supernatant carefully to a new 50 mL centrifuge tube and centrifuge the tubes at 3,000 - 6,000 × g for 10 min to recover cell pellet.

1.7. (Optional) Discard the supernatant and wash the pellet by re-suspension in 5 mL of Buffered Peptone Water (BPW) and centrifuge the tubes at 3,000-6,000 × g for 10 min.

Note: BPW can prepared by dissolving 10 g of peptone, 5 g of sodium chloride, 3.5 g of disodium phosphate, and 1.5 g of monopotassium phosphate into 1 L of distilled water. The final pH should be adjusted to 7.2 ± 0.2 at 25 °C. Autoclave at 121 °C for 15 min. Commercially available BPW can also be used.

1.8. Discard supernatant and re-suspend the pellet in 5 mL of BPW.

2. Immunomagnetic Separation (IMS) and Multiple Displacement Amplification (MDA)

Note: To prevent cross-contamination between samples, work in a biosafety cabinet, change pipette tips for each tube, avoid touching the tube with the pipette, and do not place tubes close together in magnetic stand during the washing step.

2.1. Thaw the sample buffer and the reaction buffer from the MDA kit on ice or at 4 °C in advance.

2.2. Mix 1 mL of re-suspended cell pellet in BPW with 20 µL of anti-*Salmonella* beads in 1.5 mL microcentrifuge tubes and place it on the rotating mixer for 30 min at room temperature.

Note: Competitive flora, fat particulates, and proteins in the resuspended pellet can interfere with IMS. Dilution of re-suspended cell pellet in BPW is helpful to reduce the loss of the bead-*Salmonella* complexes from the magnetic stand during washing steps.

2.3. Insert the tubes into the magnetic stand and allow 3 min for the proper recovery of beads by inverting the rack several times to concentrate the beads into a pellet on the side of the tube.

2.4. Keep the tubes on the magnetic stand. Aspirate and discard the supernatant from each tube, as well as the remaining liquid in each tube's cap.

Note: Be careful not to disturb the pellet of IMS beads on the side wall of the tube against the magnet.

2.5. Remove the tube holder from the magnetic stand and add 1 mL of wash buffer (PBS containing 0.05 % (v/v) polysorbate 20) and invert several times to remove non-specifically binding bacteria from the complex.

2.6. Repeat steps 2.3 - 2.5 twice.

2.7. After the 3rd wash, perform the final magnetic separation of beads by repeating steps 2.3 - 2.4. Remove tubes from magnetic rack and place them in a microfuge for 1 s to spin down beads. Then, place the tubes in the magnetic rack for 3 min before removing any residual wash buffer.

2.8. Re-suspend the bead-*Salmonella* complexes in 9 µL of sSample buffer and incubate at 95 °C for 3 min for denaturation.

2.9. After cooling to 4 °C on ice, combine with 9 µL of Reaction buffer plus 1 µL of enzyme mix and keep on ice.

2.10. In a thermal cycler, incubate tubes at 30 °C for 2 h for amplification, followed by heating at 65 °C for 10 min to inactivate the enzyme and cool to 4 °C on ice.

2.11. Assess the quantity and quality of the MDA products by measuring the DNA concentration and purity (260/280 ratio > 1.8) on a fluorospectrometer instrument.

Note: Because of non-specific binding of IMS beads, genomic DNA from organisms other than *Salmonella* is likely to be present in the MDA products, but it does not affect downstream analysis.

2.12. Store the final products (20 µL) at -20 °C until use for real-time PCR and/or library preparation for quasimetagenomics sequencing.

3. Real-Time PCR

Note: This step is optional for 1) detecting *Salmonella* without subtyping, and 2) assessing sample quality prior to quasimetagenomics sequencing.

3.1. Prepare 18 μL of PCR mixture per sample, containing Universal PCR Master Mix (10 μL), forward primer (2 μL , 900 nM), reverse primer (2 μL , 900 nM), probe (2 μL , 250 nM), and molecular grade water (2 μL).

Note: *Salmonella*-specific oligonucleotide primers (forward: CTCACCAGGAGATTACAACATGG, reverse: AGCTCAGACCAAAAGTGACCATC) and probe were designed to amplify a 94-bp sequence within the *ttr* gene (GenBank accession no. AF 282268)¹³.

3.2. Mix the MDA product from step 2.12 by gently pipetting up and down bead-*Salmonella* complexes along with liquid to create a suspension. Add 2 μL of the suspension into 18 μL of PCR mixture.

3.3. Run real-time PCR using an optimized real-time PCR protocol, specifying two holding periods, one at 50 °C for 2 min and another at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

3.4. Calculate the threshold cycle (Ct), which is the number of cycles required for the fluorescence from the amplified DNA to cross the threshold line.

Note: The threshold line is set in the linear phase between base line and plateau of the amplification plots of samples. Negative results correspond to Ct values over 40 or samples with Ct values higher than that of negative control.

4. Library Preparation for Quasimetagenomic Sequencing

Note: The MDA products can be sequenced by both short read and long read (nanopore) sequencing platforms. Use the latest version of DNA library prep kits provided by the manufacturers of the sequencing platforms. Perform DNA library preparation according to manufacturers' instruction. Use the 2D low-input genomic DNA protocol for library preparation for the long-read sequencing platform⁵. For library preparation for the short-read sequencing platform, minor modifications to the manufacturer's protocol are provided below.

4.1. Transfer 40 μL of MDA products to a new plate and add 20 μL of PCR purification beads to each well.

4.2. Incubate the plate at room temperature for 5 min without shaking.

4.3. Wash the beads with 80% ethanol and dry the beads for 12 min.

4.4. Re-suspend dried beads in 53 μL of resuspension buffer and incubate for 2 min without shaking.

4.5. Dilute and pool libraries following manufacturer's instruction.

Note: A 10 pM pooled and denatured library is now ready to be sequenced.

REPRESENTATIVE RESULTS:

Prior to quasimetagenomic sequencing, the overall quantity and purity of IMS-MDA products can be evaluated by fluorospectrometer (**Table 1**).

[place **Table 1** here].

Targeted quantity assessment of *Salmonella* DNA can be performed by real-time PCR (**Table 1** and **Figure 2**), which also serves as an alternative for *Salmonella* detection if subtyping is not needed.

[place **Figure 2** here].

Fluorospectrometer readings allow initial evaluation of sample preparation. Concentration of IMS-MDA products prepared from *Salmonella* contaminated chicken breast samples ranged from 701.54 to 945.86 ng/μL, suggesting that the sample DNA has been amplified. According to manufacturer's guidance, the minimum DNA sample concentration for short read and long read sequencing is 0.2 ng/μL and 1 μg, respectively. The 260/280 ratio ranged from 1.85 to 1.88 (**Table 1**), showing high purity of the DNA samples with low levels of protein contamination.

Lower Ct values indicate higher concentrations of *Salmonella* genomic DNA in the sample. As shown in **Table 1** and **Figure 2**, Ct values of IMS-MDA products decrease as enrichment time increases. In our previous study⁴, when Ct values were lower than 25, the majority (>50%) of the SE genome was likely to be sequenced, and serotype prediction from raw sequencing reads was successful.

Unsuccessful culture enrichment or IMS can result in a relatively high Ct values indicating low concentration of *Salmonella* genomic DNA in the final sample as shown in **Figure 2**. This can happen despite a high DNA concentration and purity of the IMS-MDA products as suggested by fluorospectrometer readings.

TABLES AND FIGURES LEGENDS:

Table 1: Quality and quantity assessment of DNA samples for quasi-metagenomic sequencing. *Salmonella* cells were inoculated in 25 g of raw chicken breast of two brands at 1 CFU/g. Inoculated samples were enriched in RV for 4, 8, and 12 h before IMS-MDA.

Figure 1: Representative food and environmental samples for quasi-metagenomics detection and subtyping of *Salmonella*. Samples are placed in sterile filter stomacher bags along with RV broth.

Figure 2: Real-time PCR for *Salmonella* detection or sample assessment for quasi-metagenomics sequencing. Amplification curves of real-time PCR of *Salmonella* DNA in IMS-

MDA products after 4, 8 and 12 h of enrichment time are shown. A sample after only 2 h enrichment is also included. *Salmonella* cells (0.27 CFU/g) were inoculated 25 g of raw chicken breast from two brands A and B.

DISCUSSION:

Because of the often-low abundance and in-homogenous presence of *Salmonella* in food and environmental samples, culture enrichment before IMS-MDA is still necessary for *Salmonella* detection and subtyping; it is therefore a critical step of the protocol. To identify optimal conditions to increase the abundance of *Salmonella* relative to sample background flora, different enrichment media may be evaluated for specific samples. According to MLG and BAM, both selective medium such as RV broth and non-selective medium such as buffered peptone water and lactose broth can be used for *Salmonella* enrichment from food samples. The use of RV at 42 °C has been evaluated in our previous study⁴ for *Salmonella* enrichment from raw chicken meat, iceberg lettuce and black peppercorns to support quasimetagenomic analysis of the pathogen. According to FDA BAM and AOAC precollaborative^{8,9} and collaborative studies^{14,15}, RV is recommended for the analysis of high microbial and low microbial load foods.

The optimal duration of culture enrichment depends on multiple factors, such as sample type, level of *Salmonella* contamination, and the physiological state of the *Salmonella* contaminant. For example, when low levels of *Salmonella* (e.g., < 1 CFU/g) cells are present in a meat sample and under refrigeration stress, longer enrichment time (e.g., > 12 h) may be needed to revive and enrich *Salmonella* cells. Sufficient abundance of *Salmonella* in the modified microbiome leads to adequate sequencing coverage of the *Salmonella* contaminant genome, which can allow strain-level single nucleotide polymorphism (SNP) typing⁴. Shorter culture enrichment (e.g., less than 12 h) is possible for rapid detection⁵ or serotyping of *Salmonella*⁴.

Higher levels of *Salmonella* contamination and/or longer culture enrichment of contaminated food samples can result in higher concentrations of *Salmonella* genomic DNA in modified food microbiomes, which lead to lower Ct values from the real-time PCR analysis. Ct values display a negative correlation with sequencing coverage of the *Salmonella* contaminant genome⁴, and therefore can be used for optimization and modification of the workflow. According to our experience, culture enrichment, especially the length of it, is often the focus of troubleshooting efforts. For example, the Ct value of the IMS-MDA product from a raw chicken breast sample was relatively high at 35.94 (**Figure 1**). This sample was inoculated with 2.5 CFU/g of SE cells and incubated in BPW at 37 °C for 2 h. Sequencing of this sample did not provide sufficient coverage of the SE genome to allow serotype prediction. In comparison, all the other samples with 4 h or longer enrichment were successfully serotyped from quasimetagenomic sequencing data (data not shown).

After sequencing, we recommend using Kraken¹⁶ to identify *Salmonella* reads for further bioinformatics analyses. CFSAN SNP pipeline¹⁷ and SeqSero¹⁸ can be used for SNP typing and serotyping prediction from raw sequencing reads, respectively.

This quasimetagenomic technique has several limitations. First, DNA concentration and purity of IMS-MDA products measured by a fluorospectrometer should be used only as a preliminary indicator of proper sample preparation. Due to the high efficiency of MDA, trace amounts of input DNA can be amplified to suffice whole genome sequencing¹⁹. Therefore, high quality and quantity DNA samples can still be generated even if culture enrichment or IMS fails to effectively concentrate *Salmonella* cells. The optional real-time PCR analysis provides a more targeted and informative assessment of the entire sample preparation workflow. Second, difficult-to-culture *Salmonella* cells, such as viable but nonculturable (VBNC) bacteria²⁰, may not grow during culture enrichment. Therefore, these cells may not be detected by our method.

In summary, compared with traditional methods, our sample preparation method and quasimetagenomic sequencing approach can allow substantial reduction of the analytical turnaround from *Salmonella*-contaminated food and environmental samples to robust subtyping of the *Salmonella* contaminants. In addition to *Salmonella*, this technique has the potential be applied to rapid and concerted detection and subtyping other foodborne pathogens.

ACKNOWLEDGMENTS:

The authors would like to thank Mark Harrison and Gwen Hirsch of the University of Georgia for kindly providing the bacterial strain and other support to this study.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

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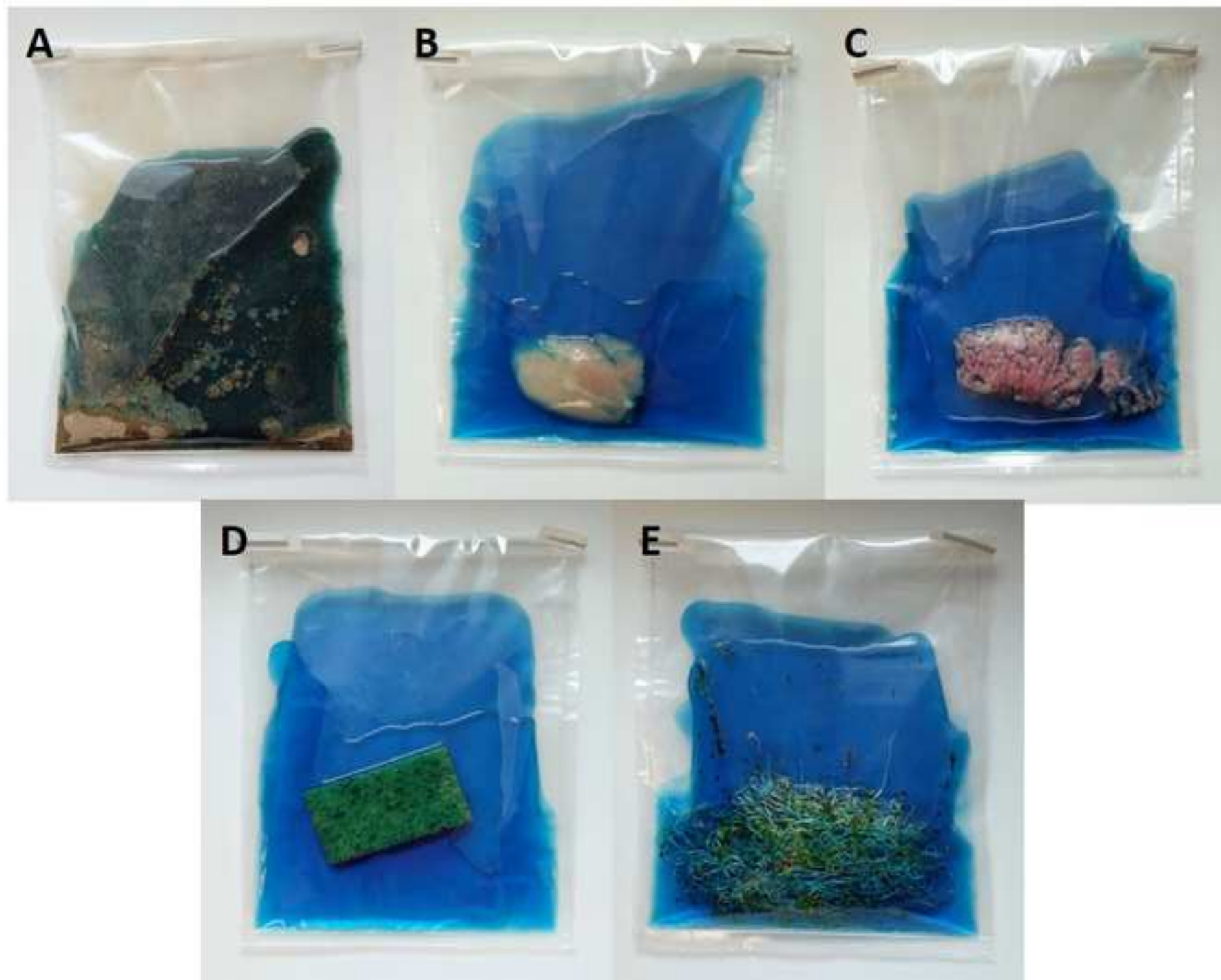


Figure 2

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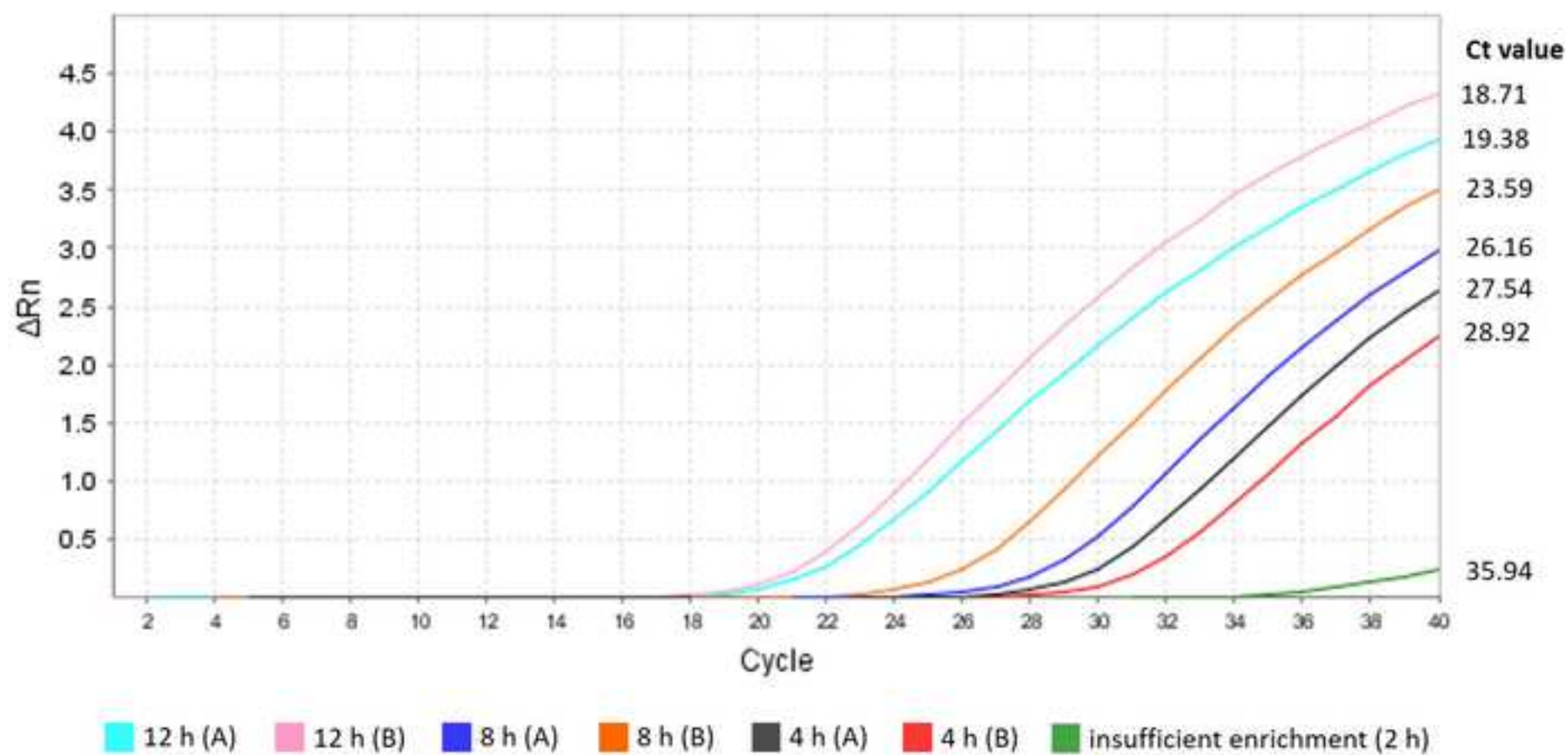


Table 1. Quality and quantity assessment of DNA samples for quasimetagenomic sequencing.

| | Enrichment time (h) | Ct value | Concentration (ng/ul) | Purity (260/280) |
|---------|---------------------|----------|-----------------------|------------------|
| Brand A | 4 | 24.71 | 812.91 | 1.89 |
| | 8 | 25.80 | 900.36 | 1.87 |
| | 12 | 15.41 | 753.88 | 1.84 |
| Brand B | 4 | 20.63 | 872.61 | 1.87 |
| | 8 | 22.61 | 993.41 | 1.87 |
| | 12 | 19.08 | 954.44 | 1.87 |

| Name of Reagent/ Equipment | Company | Catalog Number |
|--|-----------------------------|-----------------------|
| Laboratory blender bag w/filter | VWR | 10048-886 |
| Buffered peptone water | Oxoid Micorbiology Products | CM0509 |
| Rappaport Vassiliadis broth | Neogen Acumedia | 7730A |
| Polysorbate 20 | Millipore Sigma | P9416 |
| Stomacher blender | Seward | 30010108 |
| Centrifuge | Fisher Scientific | 75005194 |
| 50ml Centrifuge tubes | Fisher Scientific | 05-539-6 |
| Thermal Cycler | Techne Prime | EW-93945-13 |
| StepOne Real-Time Thermal cycler | Applied Biosystems | 4.76357 |
| AMPure XP beads | Beckman Coulter | A63881 |
| Nextera XT library prep kit | Illumina | FC-131-1024 |
| Minlon library prep kit | Oxford Nanopore | SQK-LSK108 |
| NanoDrop | Thermo Scientific | ND-2000 |
| Dynabead Anti-Salmonella beads | Applied Biosystems | 71002 |
| Illustra GenomiPhi V2 DNA amplification kit (MDA kit) | | |
| -Sample buffer | | |
| -Reaction buffer | | |
| -Enzyme mix | GE Healthcare | 25-6600-30 |
| HulaMixer | Invitrogen | 15920D |
| DynaMag magnetic rack | Invitrogen | 12321D |
| TaqMan Universal PCR mastermix | Applied Biosystems | 4304437 |
| Microfuge | Fisher Scientific | 05-090-100 |

Comments/Description

Tween 20

PCR purification beads; mix well before use; store at 4C

Store at -80C

Store at -80C

Vortex well prior to use

Store at -80C

Mix well before use; store at 4C



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Sample preparation for quasimetagenomic analysis of Salmonella from food and environmental samples

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Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to ...”

We revised the Short Abstract as suggested.

Please revise and expand the Introduction to include all of the following:

- a) A clear statement of the overall goal of this method
- b) The rationale behind the development and/or use of this technique
- c) The advantages over alternative techniques with applicable references to previous studies
- d) A description of the context of the technique in the wider body of literature
- e) Information to help readers to determine whether the method is appropriate for their application.

We believe items a) and b) have been adequately covered:

- a) A clear statement of the overall goal of this method

“The overall goal of this method is to prepare quasi-metagenomic DNA from food samples to allow targeted concentration of *Salmonella* genomic DNA and subsequent detection and subtyping of the *Salmonella* contaminant by sequencing.”

- b) The rationale behind the development and/or use of this technique.

“Metagenomics sequencing theoretically allows concerted detection and subtyping of foodborne pathogens. However, food samples present challenges to the pathogen analysis by direct sequencing of the food microbiome. First, foodborne pathogens are often present at low levels in food samples. Most of the commercially available rapid detection methods still require 8 - 48 h culturing to enrich pathogen cells to a detectable level¹. Second, many foods contain abundant microflora cells and/or food DNA, making foodborne pathogen DNA a small fraction of food metagenome and an elusive target for detection and subtyping by direct metagenomic sequencing. ”

We revised the INTRODUCTION to address c) and e)

- c) The advantages over alternative techniques with applicable references to previous studies:

“Compared to standard methods for *Salmonella* detection^{8,9} and subtyping¹⁰, the quasimetagenomic approach can substantially shorten the turnaround time from contaminated food and environmental samples to molecular subtypes of the pathogen by unifying the two typically separated analyses into a single workflow.”

- e) Information to help readers to determine whether the method is appropriate for their application.

“This method is particularly useful for applications such as foodborne outbreak response and other trace back investigations where robust pathogen subtyping is required in addition to pathogen detection and rapid analytical turnaround is important.”

We don't understand item d) "d) A description of the context of the technique in the wider body of literature". Please explain and be specific. Do mean by some sort of primacy claim (e.g., this method being the first of its kind)?

Please move the weblinks to the Reference section and cite them here using intext citations.

We revised as suggested.

Citation?

Please note we pointed out "theoretically". To our knowledge, there is no paper that reports concerted detection and subtyping of foodborne pathogens using metagenomics sequencing, which is only theoretically possible but highly unlikely. That's why we developed the quasi-metagenomics approach as an alternative. We don't think a citation here is available.

What food sample is used here? How do you make environmental swabs? Please write the protocol being as specific as you can with respect to your experiment using imperative tense throughout.

We added representative food samples used in this paper. We added how to prepare environmental swabs.

This is commercial. Please use generic term instead. Maybe sterile lab blender bags. Also Please refer to the commercial terms in the table of materials.

We changed the term as suggested. We added the commercial term to the table of materials.

This is commercial. Please use generic term instead. Maybe lab blender.

Also Please refer to the commercial terms in the table of materials.

We changed the term as suggested. We added the commercial term to the table of materials.

Please expand. Also if this is commercial please use generic terms.

MLG and BAM have previously been defined and cited (see below). We don't think we need to expand again.

"Note: Food samples are prepared for pre-enrichment according to Microbiology Laboratory Guidebook (MLG) of U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) and Bacteriological analytical manual (BAM) of U.S. Food and Drug Administration (FDA)"

What and how? In which container. Please provide details.

We added details as suggested.

Tube or tubes ? You always get 50 ml samples or more?

We changed “tubes” to “tube”. Yes, as stated in the protocol, always take a 50 mL sample.

Place tube close together or do not place it close together?

We added “do not”.

The note fits here better. Moved under this step. Please check.

We agree and confirm.

Please use generic term

We changed it to polysorbate 20.

So, after repeating the steps 2.3 – 2.5 you again repeat 2.3 to 2.4? This clarity needs to be brought out

That’s correct. 2.3 and 2.4 together allow magnetic separation of beads. After the third wash, this is needed. We clarified this step as follows.

After the 3rd wash, perform the final magnetic separation of beads by repeating steps 2.3. - 2.4.

Please provide the speed in x g

We changed the unit.

How do you avoid the non- specific binding?

Non-specific binding is not avoidable and does not affect downstream sequencing and analysis. Non-specific binding is the reason why this method is called quasimetagenomics. We added a note to clarify.

Please provide the primer sequence as well.

We added primer sequences.

Along with the beads? Please provide the step number.

Yes. We clarified this step as follows.

“3.2. Mix the MDA product from 2.10 by gently pipetting up and down bead-*Salmonella* complexes along with liquid to create a suspension. Add 2 µL of the suspension into 18 µL of PCR mixture.”

We cannot have three instances of note together. Please consider moving some details to the discussion section. Please have one note at one place.

We combined two notes and changed a note to main text.

Fluorospectrometer or spectrofluorometer ?

Fluorospectrometer is the generic name for Nanodrop.

<https://www.thermofisher.com/order/catalog/product/ND-3300>

As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

“Because of the often-low abundance and in-homogenous presence of Salmonella in food and environmental samples, culture enrichment before IMS-MDA is still necessary for Salmonella detection and subtyping; **it is therefore a critical step of the protocol.**”

b) Any modifications and troubleshooting of the technique

Paragraph 4 of DISCUSSION is dedicated to troubleshooting, including the discussion of a specific example. We revised this part to make it more explicit.

“Higher levels of Salmonella contamination and/or longer culture enrichment of contaminated food samples can result in higher concentrations of Salmonella genomic DNA in modified food microbiomes, which lead to lower Ct values from the real-time PCR analysis. Ct values display a negative correlation with sequencing coverage of the Salmonella contaminant genome⁴, **and therefore can be used for optimization and modification of the workflow.** According to our experience, culture enrichment, especially the length of it, **is often the focus of troubleshooting efforts.** **For example,** the Ct value of the IMS-MDA product from a raw chicken breast sample was relatively high at 35.94 (Figure 1). This sample was inoculated with 2.5 CFU/g of SE cells and incubated in BPW at 37 °C for 2 h. Sequencing of this sample did not provide sufficient coverage of the SE genome to allow serotype prediction. In comparison, all the other samples with 4 h or longer enrichment were successfully serotyped from quasimetagenomic sequencing data (data not shown).”

c) Any limitations of the technique

“**The quasimetagenomic technique has several limitations.** First, DNA concentration and purity of IMS-MDA products measured by fluorospectrometer should be used only as a preliminary indicator of proper sample preparation. Due to the high efficiency of MDA, trace amounts of input DNA can be amplified to suffice whole genome sequencing¹⁷. Therefore, high quality and quantity DNA samples can still be generated even if culture enrichment or IMS fails to effectively concentrate Salmonella cells. The optional real-time PCR analysis provides a more targeted and informative assessment of the entire sample preparation workflow. Second, difficult-to-culture Salmonella cells such as so called viable but nonculturable (VBNC) bacteria¹⁸ may not grow during culture enrichment. Therefore, these cells may

not be detected by our method.”

d) The significance with respect to existing methods

“In summary, compared to traditional methods, our sample preparation method and quasi-metagenomic sequencing approach can allow substantial reduction of the analytical turnaround from Salmonella-contaminated food and environmental samples to robust subtyping of the Salmonella contaminants.”

e) Any future applications of the technique

“In addition to Salmonella, this technique has the potential be applied to rapid and concerted detection and subtyping other foodborne pathogens.”

Is this open access?

Yes.

Are this open access?

Yes.