JAN 25, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.e6nvwkxp7vmk/v1

Protocol Citation: Rebecca P Wilkes, Jobin J Kattoor 2023. Meat speciation NGS protocol using DNeasy mericon food kit, Ion AmpliSeq Library Kit Plus, Ion Chef, and Ion GeneStudio S5 System. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvwkxp7vmk/v1

Protocol status: Working
We use this protocol and it's working

Created: Aug 12, 2022

Last Modified: Jan 25, 2023

PROTOCOL integer ID:
68562

Meat speciation NGS protocol using DNeasy mericon food kit, Ion AmpliSeq Library Kit Plus, Ion Chef, and Ion GeneStudio S5 System

Rebecca P Wilkes[1], Jobin J Kattoor[1]

[1]Indiana Animal Disease Diagnostic Laboratory at Purdue University

Vet LIRN

Rebecca P Wilkes
Indiana Animal Disease Diagnostic Laboratory at Purdue Unive...

DISCLAIMER

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

ABSTRACT

The purpose of this protocol is to provide the standard operating procedures for determining meat species in a food sample. This is a targeted next generation sequencing (tNGS) panel that uses manual extraction and library prep and the Ion Chef and the Ion GeneStudio S5 for sequecing. The targeting is done through PCR using primer sets in two separate pools that target multiple mitochondrial genes of various animal species, which can be purchased from ThermoFisher Scientific. This panel is useful for speciating meat in composite samples that likely contain more than one meat species. The reference and bed files needed for sequence evaluation are provided. There is some cross-detection with this panel, so evaluation of the sequencing reads obtained using BLAST analysis is recommended to confirm the results.

GUIDELINES

Wear a lab coat and gloves. Change gloves between extraction and library prep steps and any time you suspect glove contamination.

Keywords: meat speciation, targeted NGS, next generation sequencing

MATERIALS

Equipment:

Biosafety cabinet
PCR cabinet
Homogenizer
Vortexer
Centrifuge with rotor for 50 mL tubes
Microcentrifuge with rotor for 1.5 ml or 2 ml tubes, capable of attaining 17,900 x g
Shaking incubator or shaking water bath capable of attaining 60°C.
Ion Chef
Ion GeneStudio S5
Thermocycler
Qubit Fluorometer
DynaMag-96 Side Magnet (or similar)
Geneious Prime (https://www.geneious.com/)or similar for sequence evaluation

Supplies:

DNeasy mericon Food kit (Qiagen)
Ion AmpliSeq Library Kit Plus (ThermoFisher Scientific)
Ion Xpress Barcode Adapters for multiplexing (ThermoFisher Scientific)
Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (ThermoFisher Scientific)
AmPure XP reagent (Beckman Coulter)
Qubit dsDNA HS Assay Kit (ThermoFisher Scientific)

gloves
lab coat
Ethanol (96–100% and 70%)
Chloroform
Low TE
Centrifuge tubes (50 ml)
Microcentrifuge tubes (1.5 ml or 2 ml)
Centrifuge with rotor for 50 ml tubes
Pipets and pipet tips

Primer pools are available from ThermoFisher Scientific

SAFETY WARNINGS

Treat samples as biohazardous- perform extraction in a BSL-2 laboratory using a biosafety cabinet. Once nucleid acid is extracted, it is safe to transfer to a PCR cabinet for library prep steps. Extraction and library prep should be performed in separate locations.

BEFORE START INSTRUCTIONS

To do prior to starting extraction:

1. Homogenize the food sample.

2. Buffer AW2 in the Mericon Food extraction kit is supplied as a concentrate. Before using it for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Prior to chip loading and sequencing:
Upload the reference and bed files provided below into the Ion S5 Torrent Server under Reference Sequences using Import Custom Reference.

Purdue_mtDNA.fasta

PURDUE_mtDNA.designed.bed

Nucleic Acid Extraction- DNeasy mericon food kit (Qiagen)

1 See information provided in Guidelines and Warnings prior to starting the procedure.

Nucleic Acid Extraction

45m

1.1 Extraction procedure: HB-0422-003_1120350_HB_DNY_mericon_Food_0220_WW.pdf

45m

1. Place approx. 2g of homogenized food sample into a 50 mL centrifuge tube and add 10 mL Food Lysis Buffer and 25 µL of Proteinase K solution. Pulse vortex for 5-10s to ensure complete disruption and moistening of the sample.

2. Incubate for 30 min at 60°C with constant shaking. Cool the sample to room temperature (15-25°C) on ice after incubation.

3. Centrifuge for 5 min at approx. 2500 x g.

4. Pipet 500 µL chloroform into a 2 mL microcentrifuge tube

5. Carefully transfer 700 µL of the clear supernatant from step 3 to the microcentrifuge tube containing the chloroform. Be sure not to carry over material from the bottom phase, which contains precipitated food debris.

6. Vortex the microcentrifuge tube from step 5 vigorously for 15 s and centrifuge at 14,000 x g for 15 min. If the supernatant is not clear, centrifuge again for 5 min.

7. Pipet 350 µL Buffer PB into a fresh 2 ml microcentrifuge tube, add 350 µL of the upper, aqueous phase from step 6 and mix thoroughly by vortexing.

8. Pipet the solution from step 6 into the QIAquick spin column placed in a 2 mL collection tube. Centrifuge at 17,900 x g for 1 min and discard the flow-through. Reuse the collection tube in step 8.

9. Add 500 µL Buffer AW2 to the QIAquick spin column, centrifuge at 17,900 x g for 1 min and discard the flow-through. Reuse the collection tube and centrifuge again at 17,900 x g for 1 min to dry the membrane.

10. Transfer the QIAquick spin column to a 1.5 mL or 2 mL microcentrifuge tube (not supplied), and pipet 150 µL Buffer EB directly onto the QIAquick membrane. Incubate for 1 min at room temperature (15–25°C), and then centrifuge at 17,900 x g for 1 min to elute.

Library Prep- Ion AmpliSeq™ Library Kit Plus

2 MAN0013432_Ion_AmpliSeq_Library_Prep_on_Ion_Chef_UG.pdf 30m

Set up DNA target amplification reaction- 2 primer pools

Component	Volume
2X 2-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	5 µL

DNA (2–100 ng), or Direct FFPE DNA preparation	≤7.5 µL
Nuclease-free Water	to 12.5 µL
5X 2-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.5 µL
DNA (2–100 ng), or Direct FFPE DNA preparation	≤13.5 µL
Nuclease-free Water	to 18 µL

2. Mix thoroughly by pipetting up and down 5 times, then transfer sample-specific master mixes to 2 wells of a96-well PCR plate:

•For 2X primer pools, transfer 5 µL of master mix into 2 wells. Add 5 µL of primer pool 1 into the first well, and 5 µL of primer pool 2 to the second well.

•For 5X primer pools, transfer 8 µL of master mix into 2 wells. Add 2 µL of primer pool 1 into the first well, and 2 µL of primer pool 2 to the second well.

3. Cover with a plate seal and Proceed to "Amplify the targets".

2.1 Amplify the targets: 3h

A	B	C	D
Stage	Step	Temp	Time
Hold	Activate the enzyme	99°C	2 minutes
Cycle	Denature	99°C	15 seconds
Cycle	Anneal and extend	60°C	4 min
Hold	—	10°C	Hold

2.2 Combine target amplification reactions. 5m
For each sample, combine the 10 µL target amplification reactions. The total volume for each sample should be ~20 µL.

2.3 Partially digest amplicons: 45m

1. **Add 2 µL of FuPa Reagent** (brown cap) to each amplified sample. The total volume is

~22 µL.

2. Seal the plate with a clear adhesive film, vortex thoroughly, then centrifuge to collect droplets.

3. Load in the thermal cycler, then run the following program:

	Temperature	Time
	60°C	10 minutes
	55°C	10 minutes
	50°C	20 minutes
	10°C	Hold (up to 1 hr)

2.4 Ligate adaptors to the amplicons and purify:

When sequencing **multiple libraries on a single chip**, you *must* ligate a **different barcode adapter** to each library. DNA and RNA libraries from the same sample also require different barcodes.

1. If there is a visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.

2. Briefly centrifuge the plate to collect the contents.

3. Carefully remove the plate seal, then add the following components in the order that is listed to each well containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and adapters can be combined before addition.

	A	B	C
	Order of addition	**Component**	**Volume**
	1	Switch Solution (yellow cap)	4 µL

	A	B	C
	2	Adapters (Ion Torrent™ Dual Barcode Adapters, IonCode™ Adapters, *or* diluted Ion Xpress™ barcode adapter mix (for barcoded libraries))	2 µL
	3	DNA Ligase (blue cap)	2 µL
	—	**Total volume**	**~30 µL**

4. Seal the plate with a new MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

5. Load in the thermal cycler, then run the following program:

	Temperature	Time
	22°C	30 minutes
	68°C	5 minutes
	72°C	5 minutes
	10°C	Hold (up to 24 hours)

2.5

Purify the library:

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.

2. Carefully remove the plate seal, then add 45 µL (1.5X samplevolume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

3. Incubate the mixture for 5 minutes at room temperature.

4. Place the plate in a magnetic rack such as the DynaMag™-96Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.

5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.

6. Repeat step 5 for a second wash.

7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes.

2.6 Amplify the library:

1. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of 1X Library Amp Mix** and **2 µL of 25X Library Amp Primers** to each bead pellet.

2. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge briefly to collect droplets.

3. Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new well or a new plate without disturbing the pellet.

4. Seal the plate with MicroAmp™ Adhesive Film, load in the thermal cycler, then run the following program:

	A	B	C
	Stage	**Temperature**	**Time**
	Hold	98°C	2 minutes
	5 cycles	98°C	15 seconds
		64 C	1 minute
	Hold	10°C	Hold

2.7 Purify the amplified Library: 15m

Perform a two-round purification process with the Agencourt™ AMPure™XP Reagent:

•**First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**

•**Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

First-round purification:

1. Tap the plate gently on a hard flat surface or centrifuge briefly to collect the contents at the

bottom of the wells, then remove the plate seal.

2. Add 25 µL (0.5X sample volume) of Agencourt™ AMPure™XP Reagent to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5times.

3. Incubate the mixture for 5 minutes at room temperature.

4. Place the plate in a magnet such as the DynaMag™–96 Side Magnet for at least 5 minutes, or until the solution is clear.

5. Carefully transfer the supernatant from each well to a new well of the 96-well PCR plate without disturbing the pellet.

Second-round purification:

1. To the supernatant from step 4 above, **add 60 µL (1.2Xoriginal sample volume) of Agencourt™ AMPure™ XP Reagent**. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

2. Incubate the mixture for 5 minutes at room temperature.

3. Place the plate in the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

IMPORTANT! The amplicons are bound to the beads. **Save the bead pellet.**

4. Add 150 µL of freshly prepared 70% ethanol to each well, then move the plate side to side in the magnet to wash the beads. Remove and discard the supernatant without disturbing the pellet.

5. Repeat step 4 for a second wash.

6. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 2–5 minutes. **Do not over dry.**

7. Remove the plate from the magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.

8. Seal the plate with MicroAmp™ Adhesive Film, vortex thoroughly, then centrifuge to collect droplets.

9. Incubate at room temperature for at least 2 minutes.

10. Place the plate in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant.

2.8 15m

1. Quantify the library with a Qubit Fluorometer and calculate the dilution factor:

a. Make a 1:200 working dilution of Qubit™ dsDNA HS reagent using the Qubit™dsDNA HSBuffer.

b. Combine 10 µL of the amplified Ion AmpliSeq™ library with 190 µL of dye reagent, mix well, then incubate for at least 2 minutes.

c. Prepare each Qubit™ standard as directed in the user guide.

d. Measure the concentration on the Qubit™ Fluorometer.

e. *(Qubit™ 2.0 Fluorometer only)* Calculate the concentration of the undiluted library by multiplying by 20. Alternatively, use the "Calculate Stock Conc." feature on your instrument

2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM (average amplicon size 175 bp, concentration in ng/mL- about 100 pM- 11)

3. Dilute the library to about 100 pM, combine, then proceed to template preparation or store libraries. You can store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

Creating Chip plan and Loading IonChef

12h 15m

3 15m

MAN0016854_510_520_530_Kit_Chef_UG.pdf

1. Keep the Ion 510,520,& 530 Reagent cartridge stored at -20ºC at room temperature for at least 30 minutes.

2. Sign in to the Torrent suite software using credentials (http://10.160.129.7/).

3. In the **Plan** tab, click on **Plan New Run.** Select **Ion Reporter Account** as "None" and **Sample Grouping** as "Other" and click on **Next**.

4. Click on "DNA" in the **Research Application** and AmpliSeq DNA in **Target Technique** and click on **Next**.

5. Set the instrument type (e.g. Ion GeneStudio^{TM} S5 System), Library kit (e.g.: Ion AmpliSeq Library kit plus) used accordingly and select IonChef.

6. In the **Template Kit**, select **Ion 510, Ion 520 & Ion 530 Kit-Chef**, and in **Sequencing Kit**, select **Ion S5 Sequencing Kit** from the drop-down menu.

7. In the **Chip Type** drop-down menu, select the chip accordingly to the number of different samples/barcodes used.

8. **Barcode Set** can be selected optionally depending on the type of barcode used.

9. In the **Flow** tab, fill in **500** and click on **Next**.

10. Select the previously uploaded Plugins for analysis, including **AssemblerSPAdes**, and **File exporter**, and click **Next**.

11. Optionally, for saving results under a specific project, a project name can be created and selected in the **Projects** window, or click on **Next**.

12. Fill in the **Run Plan Name** and already uploaded **Reference Library-Purdue_mtDNA.fasta (mitochondrial DNA file)** and **Target regions-PURDUE_mtDNA.designed.bed** from the drop-down menu.

13. Fill in the required **Number of barcodes** and **Chip Barcode** (9-digit alphanumeric barcode).

14. Fill in the corresponding **Barcode** and **Sample Name** in the following table and click on **Plan Run**.

15. Repeat Step 1 of Section 3 to create a plan for the second chip.

3.1 12h

1. At least 30 minutes after keeping the Ion 510,520,& 530 Reagent cartridge at room temperature, proceed with the following steps.

2. Add the diluted barcoded library pools from step 3 of section 2.8 into positions 1 and 2 of the Ion S5 Reagent cartridge. Position 1 of the Ion 510,520,& 530 Chef Reagents cartridge corresponds to the libraries planned in Chip 1, and position 2 corresponds to those samples planned in Chip 2. (Figure. 1).

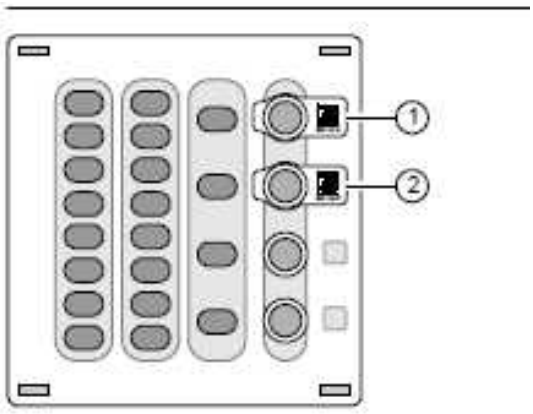

Figure 1: Position of sample and reagent tubes in Ion 510, 520, & 530 reagent cartridge. 50µL of pooled barcoded libraries are added into position A and B of the cartridge.

3. Click on the open symbol on the IonChef screen to open the door. Keep all the reagents and consumables as shown in Figure 2 following the on-screen prompt on IonChef.

4. Uncap all the tubes in the Ion 510,520,& 530 Chef Reagents cartridge.

5. Close the door after keeping the reagents and other consumables and proceed for **Deck Scan**.

6. After completing Deck Scan successfully, click **Next** and select the specific plans created in section 3 for each Chip; Chip 1 and Chip 2. In case of no plans are shown on the screen, click **Refresh Plans** and select the plans once they are shown. Click **Next** once the plan is selected.

7. On the next screen, click **Timer** and select the time to end the Chip loading function on IonChef. On completion, the chip will be stored in IonChef at 4ºC for a maximum of 24 hours. Otherwise, when the run is complete, unload the Ion Chef™ Instrument. Once taken out from the IonChef, and if two chips were loaded at the same time, the first chip goes directly into the sequencer for sequencing, and the second chip can be stored in a chip container and **keep at 4ºC** until use.

8. After unloading the used consumables except for the empty pipette tip holder (which will be moved from position 3 to position 1 as in Figure 2) from the IonChef, close the door of the IonChef and proceed with the **Clean instrument** function when prompted.

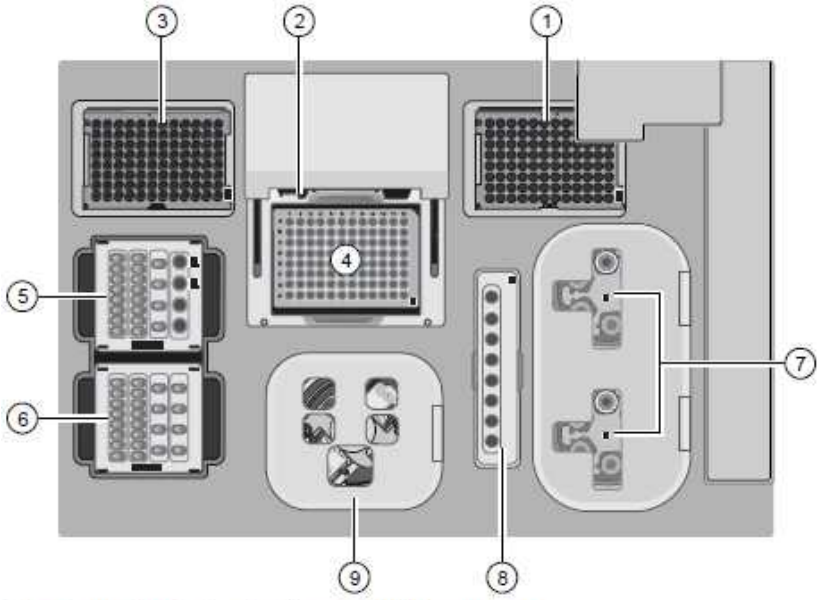

Figure 2: Position of consumables on IonChef™ for Ion chip loading

Sequencing using Ion GeneStudio S5

7h

4 1. Each initialization with the Ion S5 sequencing kit is suitable for sequencing two chips (two sequencing runs). The Ion **S5 sequencing cartridge** should be brought to room temperature at least **2 hours prior**, and **initialization** should start at least **1 hour before** the end of Chip loading described in step 7 of section 3.1.

2. To start the initialization of the sequencer, click on **Initialize** on the home screen of the Ion S5 sequencer.

3. Remove the used Ion S5 wash solution bottle and empty the wash buffer waste tank.

4. Place a new Ion S5 wash solution and an Ion S5 cleaning solution in the designated positions.

5. Place the thawed Ion S5 Sequencing cartridge into the position in the sequencer.

6. Place a used chip on the chip holder, close the sequencer door, and start initialization.

7. Initialization will take approx. 45 minutes, and when completed, click on **Home** to open the **Run** option.

8. Click **Run** and proceed as prompted on the screen of the Ion S5 sequencer. Replace the chip on the sequencer with the new Chip (Chip 1) from step 7 of section 3.1, close the door of the sequencer and proceed as prompted.

9. Click on the correct plan from the drop-down menu, uncheck the **Enable post-run clean**, and tap **Review**.

10. Tap **Start** if the plan is shown correctly for the selected chip.

11. First chip sequencing will take 2h 30 mins and keep the second chip **at room temperature** at least **30 minutes** prior to the end of first chip sequencing.

12. When the first run is finished, click on **Run complete** to open the dialogue window for the next run.

Analysis using Torrent suite software and Geneious Prime

4h

5

4h

1. Open the Torrent Suite Software (TSS) (http://10.160.129.7/) and in the **Data** tab, click on the plan name described in section 3.15, which was created for running the sequencing.
2. Aligned, trimmed files available as BAM files from the TSS can be downloaded into the local drive and can be opened in Genious prime software (https://www.geneious.com/).
3. Based on the alignment to reference sequences in the TSS, sequence reads can be viewed in the Genious prime software.
4. For additional confirmation, each sequence can be subjected to a BLAST analysis at https://blast.ncbi.nlm.nih.gov/ for the Percent identity with species of interest.