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Prospecting for zoonotic pathogens using targeted DNA enrichment

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We are still developing and optimizing this protocol

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Abstract

There are over 60 zoonoses linked to small mammals, including some of the most devastating pathogens in human history. Meanwhile, millions of museum-archived tissues are available to understand natural history of these pathogens. Our goal is to maximize the value of museum collections for pathogen-based research using targeted sequence capture. To this end, we have generated a probe panel that includes 39,916, 80bp RNA probes targeting 32 pathogen groups, including bacteria, helminths, fungi, and protozoans. Laboratory generated, mock control samples show that we are capable of enriching targeted loci from pathogen DNA 2,882 to 6746-fold. Further, we were able to identify bacterial species inmuseum-archived samples, including *Bartonella*, a known human zoonosis. These results show that probe-based enrichment of pathogens is a highly customizable and efficient method for identifying pathogens from museum-archived tissues.

Guidelines

Aseptic Protocols

Wear disposable gown over lab coat during DNA extraction process.
Clean work surfaces with 3% sodium hypochlorite solution.
Aliquot all reagents under a Biological Safety Cabinet.
Single use of reagents aliquot on each work day.
Space out samples by leaving one empty rack space between Microcentrifuge tubes.
Process a sample at a time and sterilize gloves with 70% ethanol between samples.

Materials

1. **Liver of naïve (uninfected) laboratory mouse *Mus mus*:** We used C57BL/6J (SN: 000664) laboratory mouse (*Mus mus*) supplied by the Jackson Laboratory (**https://www.jax.org/**) and kept in the Texas Biomedical Research Institute vivarium. The mouse was not exposed or infected with any of our control pathogens.

2. **Pathogens DNA:** *Mycobacterium* and *Plasmodium vivax* DNA were provided by Dr. Larry Schlesinger and Dr. Ian Cheeseman laboratories at Texas Biomedical Research Institute. *Plasmodium falciparum*, *Schistosoma mansoni*, and *S. bovis* DNA were from Dr. Timothy Anderson Malaria and schistosome laboratories.

3. **Natural Science Research Laboratory museum samples:** A total of 38 samples of mammalian liver tissues (10 tissues lysed in buffer and 32 tissues frozen in liquid nitrogen) collected between 1996 and 2009 in Africa, North America and the USA (see Table 1 in publication).

4. **Tubes:** 1.5 mL microcentrifuge tubes, 1.5 mL DNA LoBind tube (Eppendorf, Cat.#022431021) and 0.65 mL PCR tubes

Reagents

1. DNA extraction (Qiagen, DNeasy® Blood and Tissue Kit): i) Buffer ATL: Lot # 5690100, ii) Buffer AL: Lot # 56902506, iii) Buffer AW1: Lot # 56903160, iv) Buffer AW2: Lot # 56903453, v) Proteinase K: Lot # 169034384.

2. NGS libraries preparation (KAPA Hyperplus kit): Ref.# 07962428001, Lot # 0000128102.
myBaits Hybridization capture for NGS at 100 ng/µL (v.5, Daicel Arbor Biosciences): Cat.# 300248R.V5, Ref.# 210401-903, Lot.# 210210.

3. NGS libraries quantification (KAPA Library Quantification Kit): Ref.#07960204001, Lot # 0000134934.

4. NGS library adapters (IDT xGen Stubby Adapter-UDI Primers, 96 rxn): Ref. # 402130088, Part # 10005921.

5. NGS library purification and size selection (KAPA Pure Beads): Ref.# 07983271001, Lot.# 0000131236.

6. NGS library quality control (Agilent Technologies D1000 Screen Tape): Part # 5067-5582, Lot.# 0202074-176.

7. Fisher Absolute Ethanol: Lot # 210850

8. Tris-HCl Buffer (1 M, pH 8.0), Cat.# BM-320, Lot.# C31N129

9. PCR grade water (Gibco Distilled Water), Ref.# 15230-170, Lot.# 2390091 and Lot.# 2318146

10. DNA quantification (Quant-iT™ dsDNA BR Assay Kit): Cat.# Q33120

Equipment

1. Biological Safety Cabinet (Baker SterilGARD® III Advance)

2. Thermo Savant ISS110 SpeedVac System Concentrator

3. Agilent 4200 TapeStation System

4. Qubit 1.0 Fluorometer

5. Applied Biosystems QuantStudio™ 5 Real-Time PCR System

6. Thermo IEC Centra CL3R Benchtop Centrifuge

7. Applied Biosystems SimpliAmp™ Thermal Cycler

8. Fisher Scientific Isotemp 120 Water Bath

9. BioRad Digital Dry Bath

10. Fisherbrand™ Variable Speed Nutator

11. Eppendorf™ 5424 Microcentrifuges

12. Corning-6765 Mini Centrifuge

13. Fisher Scientific Digital Vortexer

14. Fisher Scientific Balance accu-413

DNA extraction protocol (slight modification from DNeasy® Blood and Tissue Kit protocol for tissues)

16h 31m 20s

1	Put ~ 10 mg – 20 mg of tissue in 1.5 mL microcentrifuge tubes (Note: for lysed tissue, put 180 µL of lysate in 1.5 mL microcentrifuge tubes and proceed to step iii)	1m
2	Add 180 µL of ATL buffer	20s
3	Add 20 µL of Proteinase K	20s
4	Vortex at 2000 rpm for 10 sec. and spin down in microcentrifuges for 10 sec.	20s
5	Put sample in Dry Bath preheated at 56°C overnight	16h
6	Vortex at 2000 rpm for 10 sec.	10s
7	Add 200 µL of Buffer AL	20s
8	Add 200 µL of Absolute ethanol	20s
9	Vortex at 2000 rpm for 3 min	3m
10	Pipet the mixture into a DNeasy Mini spin column placed in a 2 mL collection tube.	20s
11	Centrifuge at 8000 rpm for 1 min. Discard the flow-through and collection tube.	1m 20s
12	Place the spin column in a new 2 mL collection tube. Add 500 µL Buffer AW1.	30s

13 Centrifuge at 8000 rpm for 1 min. Discard the flow-through and collection tube. 1m 20s

14 Place the spin column in a new 2 mL collection tube, add 500 µL Buffer AW2 20s

15 Centrifuge at 14,000 rpm for 3 min. Discard the flow-through and collection tube 3m 20s

16 Transfer the spin column to a new 1.5 mL microcentrifuge tube. 20s

17 Elute the DNA from the spin column by adding 50 µL of 10 mM Tris-HCl, pH 8.0 (pre heated at 56°C for 10 min.) 1m

18 Incubate for 5 min at room temperature 5m

19 Centrifuge at 8000 rpm for 1 min, discard spin column and quantify DNA with Qubit Fluorimeter using the Quant-iT™ dsDNA Broad Range Assay. 12m

NGS libraries preparations (slight modification from KAPA Hyperplus kit and combined with IDT xGen DNA Library Prep. EZ kit protocols) 2h 59m 5s

20 Put 500 ng DNA per sample in 0.65 mL PCR tubes 20s

21 Add enzymatic fragmentation reagents (KAPA Hyperplus protocol) 2m

22 Mix by pipetting and spin down for 5 sec 8s

23 Incubate at 37°C for 10 min in thermocycler (KAPA Hyperplus protocol) 11m

24 Add End-repair and A tailing reagents (KAPA Hyperplus protocol) 39s

25 Mix by pipetting and spin down for 5 sec. 8s

26 Incubate at 65°C for 30 min in thermocycler (KAPA Hyperplus protocol) 30m

27 Add Adapter ligation reagents (KAPA Hyperplus protocol)) Note: IDT xGen Stubby Adapter (from the IDT xGen Stubby Adapter-UDI Primers kit) is added to the reaction (replacing the KAPA Adapter stock) to make the required reaction volume. 2m

28 Mix thoroughly by pipetting and spin down for 10 sec. 15s

29 Incubate in at 20°C for an hour in thermocycler (Note: the lid temperature should be turned off) 1h

30 Post ligation cleanup (KAPA Hyperplus protocol). Note: mix beads and libraries by pipetting. 10m

31 Elute DNA library in 25 µL 10 mM Tris-HCl, pH 8.0 2m

32 Add 20 µL of ligated library in 0.65 mL PCR tubes 20s

33 Add Library Amplification reagents (i.e. KAPA HiFi HotStart ReadyMix (2X) from the KAPA Hyperplus kit) Note: IDT xGen UDI Primers (from the IDT xGen Stubby Adapter-UDI Primers kit) replaces the KAPA Library Amplification Primer Mix (10X) in the reaction. 1m

34 Mix thoroughly by pipetting and spin down for 10 sec 15s

35 Place tubes in thermocycler for 4 cycles of library PCR amplification (KAPA Hyperplus protocol). 12m

36 Post library amplification cleanup (KAPA Hyperplus protocol). Note: mix beads and libraries by pipetting. 10m

37 Elute amplified DNA library in 53 µL 10 mM Tris-HCl, pH 8.0. Note: use 50 µL of amplified DNA library for first DNA library size selection. 2m

38 Perform first DNA library size selection (KAPA Hyperplus protocol) by mixing 50 µL of amplified DNA library and 25 µL of KAPA Pure Beads. Note: mix beads and libraries by pipetting. 10m

39 Perform second DNA library size selection (KAPA Hyperplus protocol) by mixing 70 µL of first size cut DNA library and 10 µL of KAPA Pure Beads. Note: mix beads and libraries by pipetting. 10m

40 Elute second size cut DNA library in 22 µL PCR grade water (recover ~20 µL of final DNA library) 5m

41 Use 1 µL of final DNA library for quality control base pairs sizes and concentration estimation (Agilent Technologies D1000 Screen Tape protocol and Agilent 4200 TapeStation®) 10m

DNA Target Capture/Hybridization (slight modification from the high sensitivity protocol of myBaits® v.5 (Daicel Arbor Biosciences) Hybridization capture for NGS. 1d 17h 15m

42 Pool between 4 to 16 DNA libraries with similar DNA concentrations together into 1.5 mL tube 5m

43 Concentrate the pool of samples to 7 µL with a speedvac vacuum concentrator (Note: if volume drops below 7 µL, make up the difference by adding the appropriate volume of PCR grade water) 1h

44 First round of enrichment at 63˚C for 18 hours (myBaits® v.5 protocol) 18h

45 15 cycles of enriched libraries PCR amplification (myBaits® v.5 protocol) 50m

46 Second round of enrichment at 65˚C for 18 hours (myBaits® v.5 protocol) 18h

47 15 cycles of enriched libraries PCR amplification (myBaits® v.5 protocol) 50m

48 Use 1 µL of enriched library for quality control base pairs sizes and concentration estimation (Agilent Technologies D1000 Screen Tape protocol and Agilent 4200 Tapestation®). 10m

49 Enriched libraries quantification (KAPA Library Quantification Kit/protocol). 2h

50 Enriched libraries combined into an equimolar pool for subsequent Illumina Hi-Seq 2500 sequencing. 20m