*RNA Extraction*

RNA was extracted from a randomly selected subset of fecal (302), throat (143), and urine (196) swabs samples in the Virology Unit at Institut Pasteur of Madagascar, with each sample corresponding to a unique individual from the field dataset. Water controls were extracted in conjunction with each unique extraction day. Extractions were conducted using the Zymo Quick DNA/RNA Microprep Plus kit (ZYMO, Irvine, CA, USA), according to the manufacturer’s instructions and including the step for DNAse digestion. Post-extraction, RNA quality was checked on a nanodrop to ensure that all samples demonstrated 260/280 ratios exceeding 2 and quantifiable concentrations. Resulting extractions were stored in freezers at -80\*C, then transported on dry ice to the Chan Zuckerberg Biohub (San Francisco, CA, USA) for library preparation and metagenomic Next Generation Sequencing (mNGS).

*Library Preparation and mNGS*

A subset of four randomly selected samples from each of three bat species was selected for additional quantification using an Invitrogen Qubit 3.0 Fluorometer and the Qubit RNA HS Assay Kit (ThermoFisher Scientific, Carlsbad, CA, USA). After quantification, 5ul of of each RNA sample, plus water control, was diluted 5X and arrayed in 96-to-384 well plate format using a BRAVO Automated Liquid-Handling Platform and unique TruSeq Index PCR Primer barcodes (Illumina, San Diego, CA, USA). Samples were subsequently prepped into libraries using the NEBNext Directional RNA Library Prep Kit (Purified mRNA or rRNA Depleted RNA protocol; New England BioLabs, Beverly, MA, USA), following the manufacturer’s instructions and according to previously published modifications (XXX). Quality and quantity of resulting individual and pooled mNGS libraries were assessed via electrophoresis with the High Sensitivity NGS Fragment Analysis Kit on a Fragment Analyzer (Advanced Analytical Technologies, Inc), the High-Sensitivity DNA Kit on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and via real-time quantitative polymerase chain reaction (qPCR) with the KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA, USA). Final library pools were spiked with a non-indexed PhiX control library (Illumina, San Diego, CA, USA). Pair-end sequencing (2 × 150 bp) was performed using an Illumina NovaSeq sequencing system (Illumina, San Diego, CA, USA). The pipeline used to separate the sequencing output into 150-base-pair pair-end read FASTQ files by library and to load files onto an Amazon Web Service (AWS) S3 bucket is available on GitHub at <https://github.com/czbiohub/utilities>.

*IDSeq*

Raw reads from Illumina sequencing were host-filtered, quality-filtered, and assembled on the IDseq (v3.2) platform, a cloud-based, open-source bioinformatics platform designed for microbe detection from metagenomic data (73), using a host background model of “bat” compiled from all publicly full-length bat genomes in GenBank. Samples were deemed “positive” for coronavirus infection if IDseq successfully assembled at least one nucleotide or protein-BLAST derived contig mapping to any CoV reference accession number. To clarify that no positives were missed from IDseq, all non-host contigs assembled in IDseq underwent offline blast against a reference database constructed from all full-length reference sequences for *Alpha-* and *Betacoronavirus* available in GenBank. Step-by-step instructions for our offline BLAST protocol can be accessed in our publically available GitHub repository at: https://github.com/brooklabteam/Mada-Bat-CoV/.