# Technical Bulletin for SARS-CoV-2 Wastewater Testing

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## I. Sample Collection and Sewershed Population

***Helena Wastewater Treatment Facility (HWTF; Information provided by Mark Fitzwater and Robert Cline):*** Wastewater samples from the HWTF are collected once a week from the inlet building post grit chamber between the hours of 6:15am on Tuesday and 6:15am on Wednesday. An Isco composite sampler is used to collect the flow passed samples from the treatment facility, which has an average inflow rate of 3.5 Million Gallons (MG) per day and receives domestic, industrial, and commercial wastewater from a population of approximately 37,125 people. The samples are collected at 4°C over the 24-hour period, after which they are transferred to a Nalgene collection bottle. To disinfect the collection bottle, the outer surface is treated with a bleach solution before being placed back at 4°C. The wastewater samples are transported by a wastewater technician to Carroll College at 9am where they are immediately processed for extraction with PolyEthylene Glycol (PEG).

***East Helena Wastewater Treatment Facility (EHWTF; Information provided by Shane Pursley):*** The EHWTF serves the area within the municipal boundaries as well as several small areas outside of the city limits within Lewis & Clark County, with an estimated population served of 2,273 people. Additionally, a very small number of septic systems are discharged through truck delivery into the wastewater facility from multiple counties within the area. On average, the inflow rate is approximately 0.97MG per day. The influent sewer monitoring is conducted according to test procedures approved under Part 136, Title 40 of the Code of Federal Regulations. Wastewater samples retrieved for the State of Montana Department of Environmental Quality (DEQ) wastewater permit requirements are the same as those used for SARS-CoV-2 testing at Carroll College. A 12-hour composite wastewater sample is collected using a Teledyne Isco Refrigerated Sampler. The Teledyne compiles a 12-hour sample by collecting a 250mL sample every hour for the given sampling day between 11am and 11pm. These mixed samples form a composite and are stored at 4°C until the following morning. The composite

sample is then transferred to a 250mL container, obtained through Energy Labs in Helena, which is sanitized per protocol. The container is then placed in a cooler and delivered to Carroll College in a manner that ensures the sample temperature does not change from 4°C by plus or minus 2°C.

**II. Sample Handing and PEG Extraction**

Upon receipt of the samples from the Helena and East Helena treatment facilities, the wastewater is placed in a biosafety cabinet within a dedicated lab room at Carroll College and disinfected with a 10% bleach solution. Each wastewater sample is divided into three 40mL aliquots in 50mL Falcon tubes (VWR) and centrifuged in 4°C conditions for 5 minutes at 3,200g (Thermo Sorvall Legend RT). Following centrifugation, the supernatants are carefully transferred to clean 50mL Falcon tubes and treated with 10mL of PolyEthylene Glycol (PEG; Abcam or Sigma Aldrich). The samples are then placed overnight on a nutator mixer (Corning) at 4°C to ensure thorough mixing of the wastewater samples with the PEG. After the overnight incubation, the samples are centrifuged in 4°C conditions for 30 minutes at 3,200g. The supernatants are subsequently discarded and the pellets resuspended in 100µL of viral resuspension solution (provided in the PEG precipitation kit). The viral suspensions are either kept at 4°C until RNA extraction or processed immediately following resuspension.

**III. RNA Extraction and Real Time PCR**

Total RNA extraction of each PEG viral suspension (3 from Helena and 3 from East Helena) is carried out using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The extracted RNAs are eluted in 30µL of nuclease-free water (provided in the kit) and quantified using a Nanodrop spectrophotometer (ThermoFisher) to assess the quantity and quality of the extracted RNAs. The RNA samples are either kept on ice or stored at -80°C until they undergo Real Time PCR (RT-PCR).

RT-PCR is performed, in duplicate, on the extracted RNAs using the iTaq Universal Probes One-Step Kit (BioRad) for a total of 6 replicates (3x biological, 2x technical) for the Helena and East Helena samples. For each 20µL reaction, 50-100ng of RNA is mixed with 10µL of 2X iTaq universal probes reaction mix, 1.5µL of Reverse Transcriptase, 1.5µL each of the N1 and N2 assay mixes from the 2019-nCoV RUO qPCR primer/probe kit (Integrated DNA Technologies), and 1.5µL of nuclease-free water. To generate the standard curve for absolute quantification of virus within the wastewater samples, serial dilutions of control genomic RNA from SARS-CoV-2 (ThermoScientific/AcroMetrix) are prepared. For our analysis, SARS-CoV-2 RNA concentrations of 2.5, 5, 10, and 20 genomic copies are used as template in RT-PCR reactions that are assayed alongside the sample reactions. These concentrations were chosen as they produce Cycle threshold (Ct) values that routinely bound the range of our wastewater samples. The

reactions are prepared in 96-well reaction plates (ThermoFisher), covered with an adhesive PCR plate seal (ThermoFisher), and centrifuged briefly in a PlateFuge Microplate Centrifuge (Thomas Scientific) before undergoing RT-PCR in a BioRad iQ5 thermocycler. All reactions undergo the following thermocycling conditions: 50°C for 10 min, 95°C for 1 min, and 45 cycles of 95°C for 10 sec and 60°C for 30 sec. The Ct values for the reactions are determined by manually adjusting the threshold line so that it bypasses background signal and falls within the early exponential phase of each fluorescent curve. These Ct values are subsequently entered into an Excel spreadsheet and analyzed as described below.

**IV. Data Analysis**

To generate a standard curve for the absolute quantification of SARS-CoV-2 within the wastewater samples, the Ct values for the SARS-CoV-2 control RNA are plotted against the $\log_{10}$ genome copy number. Linear regression is then performed to generate a trend line equation (Ct = (slope) x ($\log_{10}$ copy number) + y intercept) that is subsequently used to calculate genome copy (gc) numbers using the Ct values of the wastewater samples. These values are then adjusted for the volume of RNA that was used in each RT-PCR reaction (variable), the total volume of RNA that was eluted following RNA extraction (30µL), the initial wastewater sample volume (40mL), and a mL to L conversion in order to generate a gc/L concentration. The mean gc/L concentrations for Helena and East Helena are graphed alongside the daily case numbers for Lewis and Clark County (LCC; accessed at: https://helenamtmaps.maps.arcgis.com/apps/opsdashboard/index.html) and submitted on a weekly basis for posting to the LCC Decision Making Dashboard (accessed under 'Disease Surveillance' at: https://www.lccountymt.gov/health/covid-19/local-covid-19-decision-making-dashboard.html). The Limit of Detection (LoD) for the testing procedure was determined to be 2.1 genomic copies (Range 1.07 – 3.8gc for 3 replicates, curve-fitting modeling method, 95% probability of LoD detection), or 315 gc/L, using the LoD Calculator script in R (Merkes et al., 2019; https://github.com/cmerkes/qPCR_LOD_Calc).

**V. References**

Merkes CM, Klymus KE, Allison MJ, Goldberg C, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Richter C. (2019) Generic qPCR Limit of Detection (LOD) / Limit of Quantification (LOQ) calculator. R Script. Available at: https://github.com/cmerkes/qPCR_LOD_Calc. DOI: https://doi.org/10.5066/P9GT00GB. Date Accessed: 09/24/20