Limit of detection for Biobot Analytics's E9L-NVAR orthopoxvirus assay in a wastewater context

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Mpox (MPX) disease is caused by the monkeypox virus (MPXV), endemic in Central and Western Africa (Tiwari et al 2023). However, MPXV began to spread beyond Central and Western Africa in 2022, and the World Health Organization (WHO) declared a global health emergency after more than 16,000 cases were reported from 75 different countries and territories (UN, 2022). The first documented MPXV infection in the United States was found in Boston, MA in May 2022, and it has since spread across all 50 states (CNBC 2022, CDC 2022). Clinical testing for MPXV is incomplete due to factors such as stigma, cost, and limited availability; thus, environmental surveillance such as wastewater testing is key for getting a full picture of the epidemic (Tiwari et al 2023).

In the fall of 2022, Biobot assessed and developed an influent-optimized, non-variola Orthopoxvirus isolation/capture protocol, along with a detection assay to enable MPXV monitoring in wastewater. The isolation and capture protocol is a large volume input variant of the Nanotrap® Microbiome A 10mL protocol (APP-077), where 34.3mL of the input influent wastewater is used and the isolated pathogens are extracted using the MagMAX nucleic acid isolation kit. To detect and quantify MPXV, Biobot performs quantitative PCR (qPCR) against the E9L-NVAR target with the probe and primers as described in the CDC non-variola Orthopoxvirus PCR test.

This whitepaper focuses around the limit of detection (LoD) estimates of our E9L-NVAR singleplex qPCR assays to help our customers interpret the results from Biobot's MPXV wastewater assay. ***In summary, internal calculations show that Biobot's E9L-NVAR wastewater assay has a 95% detection probability at approximately 1.15 copies per mL of wastewater input. Additional analyses of wastewater measurements suggest that the LoD in wastewater matrices is closer to 1.9 copies/mL, similar to our estimates using a synthetic template.***

In-vitro validation of LoD using synthetic standards

To quantify MPXV, we targeted an orthopoxvirus DNA polymerase gene, E9L, using a singleplex TaqMan-based qPCR assay. The LoD for our assay was determined using an MPXV synthetic linearized plasmid quantified via digital droplet PCR (NIST product page). The linearized plasmid was diluted in molecular grade water to approximately the following gene copies/well: 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0. We selected this range based on preliminary experiments that showed that our assay is able to detect one or several copies of a synthetic MPXV template. Each solution was assayed across eight technical replicates on two independent 96-well plates and cycled at the conditions in Table 1. Cycle thresholds (Cts) were determined using the Cy0 algorithm (Guescini et al 2008). All replicates with a Ct < 40 were considered detects and were included in the analysis below.

Step	Cycles	Temperature	Time (min:sec)
1	1	50°C	10:00
2	1	95°C	0:30
3	45	95°C	0:03
		60°C	0:30

The experiment was performed in duplicate. To validate our experimental results, we plotted input copies vs. calculated copies of our template, imputing nondetects as 0 copies (Fig. 1). We saw high linearity and correspondence between input copies and calculated copies, and used the combined data from both experiments to calculate the LoD. The LoD of an assay is defined as the concentration at which the assay is able to detect a target with 95% probability, and a detection of an assay was defined as 1 out of 3 wells in a 3-well replicate assay yielding a Ct <40 (Klymus et al 2019).

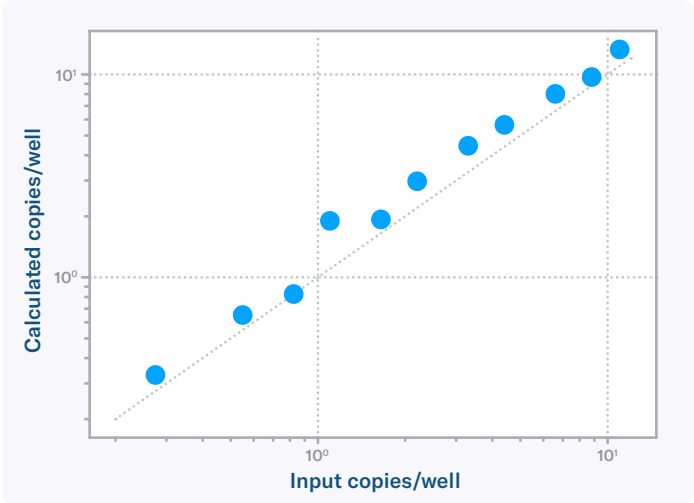

Fig. 1. Calculated average copies per well from the synthetic spike-in experiment, as a function of known input copies. Nondetects were assigned a value of 0 in the averaging.

To determine the LoD, we first estimated the probability of an individual replicate to detect X copies of the template (Fig. 2). Using this probability, we analytically derived the number of copies-per-uL that would be needed to obtain 95% detection probability of an assay (Fig. 3), similar to methods used in Klymus et al 2019. This resulted in an LoD of 1.26 copies per well.

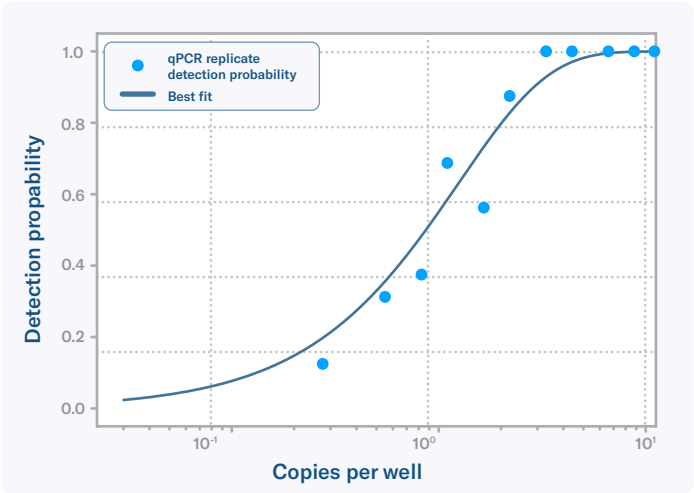

Fig. 2 Replicate detection probability as a function of input copies per well is shown as blue dots. Dark blue line represents the best fit to the function (1 - exp(-copies / A)).

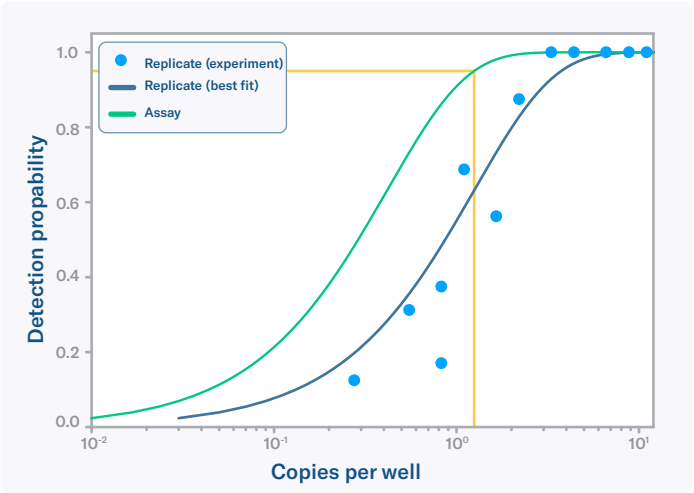

Fig 3. Illustration of the assay LoD calculation. Dark blue line from Fig 2 shows the qPCR replicate detection probability. Using the replicate detection probability, we calculate the assay detection probability shown as a green line. The concentration at which this probability is 95% is the LoD of an assay (shown as yellow lines)

To estimate the LoD in copies per mL of wastewater input, we used simple mass balance to convert LoD from copies per well to copies per mL wastewater (Fig. 4). The mass balance equation takes into account the total wastewater input, a subsampling factor during the extraction protocol, and total volume of nucleic acid (NA) used compared to the final isolated nucleic acid volume. Using this equation, we converted our LoD of 1.26 copies/well into a LoD of 1.15 copies/mL of input wastewater (Fig. 4).

$$\frac{1.26\ copies \times \frac{75uL\ total\ NA\ isolated}{3uL\ NA\ per\ input} \times \frac{500uL\ total\ extraction}{400uL\ extraction\ used}}{34.3mL\ total\ wastewater\ input} = 1.15\ copies/mL$$

Fig. 4 Transformation equation for converting LoD at copies per qPCR well back to copies per mL of wastewater.

Validation of LoD with real-world wastewater data

Biochemical reactions are known to behave differently in wastewater due to the contaminants and inhibitors present in the wastewater matrix. Therefore, we tested whether detection probability in wastewater matched our expectations from the experiment we conducted. Using MPXV wastewater data from 67 locations, we plotted how the fraction of positive E9L-NVAR replicates in an assay depended on the mean copies-per-mL inferred from the same reaction (Fig. 5). With this method, we estimated the LoD to be 1.57 copies/mL wastewater, which was similar to the experimentally obtained value of 1.15 copies/mL.

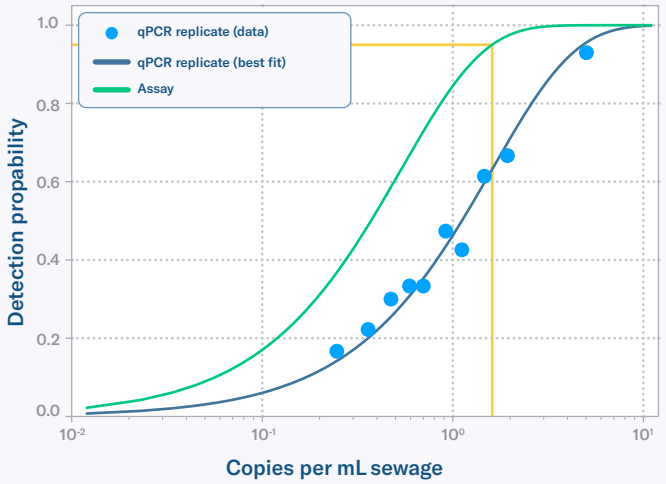

Fig. 5. *Empirically derived LoD. Blue dots represent the replicate detection probability as a function of average replicate concentration, derived from production MPX samples. Each dot is an average of 1/10 of the available samples. Dark blue line denotes the best fit to the same functional form as in Fig 2. Green line shows the assay detection probability as in Fig 3.*

We note that this method is expected to be slightly biased, because non-detects were intentionally omitted. For a synthetic assay, the exact input copies of the synthetic MPXV templates are known; thus, all nondetects were included in the analysis. Here, we only included samples that have at least one replicate detected, since we have no knowledge about copies-per-mL-wastewater for samples that are nondetects. To double check our assumptions, we performed a computer simulation of 1,000 locations with varying concentrations of MPXV, a simulated qPCR assay with a known LoD, and estimated the LoD using the method described above. Depending on the exact parameter values, the LoD can be overestimated by up to 15%. This suggests that the our calculated LoD using real-world wastewater data is between 1.6 and 1.9 copies per mL sewage.