Countable Labs. UNIVERSITY OF GEORGIA

Multiplexed single-tube detection of *Trypanosoma cruzi* DNA with million-fold dynamic range using single-molecule PCR

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Abstract

Chronic *Trypanosoma cruzi* infection, known as Chagas disease, is a major cause of cardiomyopathy in the Americas. Detection of *T. cruzi* infection is complicated by low parasitemia, which often evades detection with conventional PCR. Deep-sampling qPCR improves sensitivity by running hundreds of replicates, but this approach is too labor- and cost-intensive for diagnostics or routine monitoring. We aimed to develop an improved PCR method for *T. cruzi* detection from blood samples that would reduce cost and labor, thereby making routine testing for Chagas disease possible.

Methods:
We evaluated a multiplexed, single molecule-based PCR platform that performs target amplification inside of ~30 million compartments in a 50 µL reaction and uses automated 3D imaging and analysis to count target-positive compartments. Using blood-extracted DNA from 45 *Macaca fascicularis* with acquired *T. cruzi* infections, we assessed detection of both satellite DNA (sDNA) and kinetoplast DNA (kDNA) across matched samples previously tested by deep-sampling qPCR.

Results:
Countable PCR matched the sensitivity of 10 to 388 qPCR replicates in a single reaction, achieving limits of detection as low as 4 copies for sDNA and 16 copies for kDNA. The system maintained high linearity across a near million-fold range of input DNA. Matched analysis showed strong correlation to qPCR Cq values, and multiplexed detection of parasite targets enhanced robustness of the assay.

Background

Counting genomes for better health outcomes

- The early stage of this disease often has mild symptoms or goes unnoticed
- Because chronic infection can lead to serious heart and digestive system issues, early diagnosis and treatment can be lifesaving
- Common in Mexico, Central and South America, and the southern United States of America

T. cruzi infections, which can persist at ultra low but harmful levels, benefit from early medical intervention when detected. Therefore, being able to detect low abundance of parasite from blood extractions is vital. Current molecular-based method for detection of circulating *T. cruzi* utilizes deep sampling with qPCR. This involves sampling a very large DNA mass, with which many qPCR replicate reactions must be performed, in some cases as many as 100+ per sample, to detect a positive parasite genome. This requires extensive resources.

Method

Targeting repetitive elements in the *T. cruzi* genome for parasite detection.

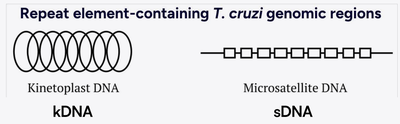


Figure 1. **Telltale parasite targets.** Assays that target repetitive elements in the parasite genome can be leveraged for improved detection sensitivity but only when elements are counted independently.

Enhancing the sensitivity of *T. cruzi* detection by digesting out repeat elements followed by single molecule counting.

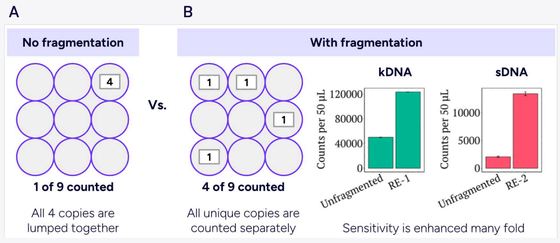


Figure 2. **The advantage of fragmentation of repetitive elements coupled with single molecule counting.** Without fragmentation, repeated elements adjacent on the genome can end up within the same compartment. (A) When counting target molecules, grouped targets become counted only once because they are lumped together. (B) Fragmenting elements allows them to individually compartmentalize so each is counted one-for-one. This lead to an up to 10-fold increase in detection sensitivity.

The process workflow involves filtration, digestion, and IAC spike-in.

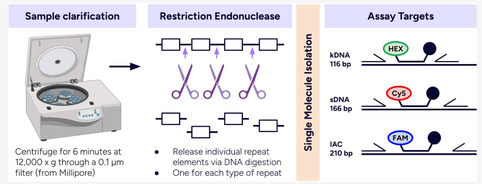
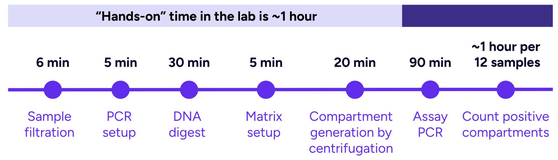


Figure 3. Sample pre-processing is done to improve single molecule isolation

Filtration was done on extracted DNA from blood samples to remove small contaminants from the DNA prep, such as residual cell debris. Extracted DNA samples were digested with a mix of restriction endonuclease enzymes to liberate the individual repeat elements as described above. The single molecule elements were detected with the 3-plex *T. cruzi* assay in Countable PCR. Counts per target were reported by Countable PCR analysis.

For samples after extraction, an internal amplification control (IAC) made from synthetic dsDNA was added at >3,000 copies per sample. IAC is used to detect PCR inhibition. Knowing the number of IAC copies added to a sample allows PCR interference to be observed when IAC counts are below expected. Samples negative for sDNA or kDNA are valid if IAC counts are correct. Else, an absence or loss in IAC means that sample cannot be fairly evaluated for parasite burden.

Fast time to results for *T. cruzi* detection in extracted blood samples.



Method, cont.

1-tube deep sampling with Countable PCR vs. 10 qPCR reactions.

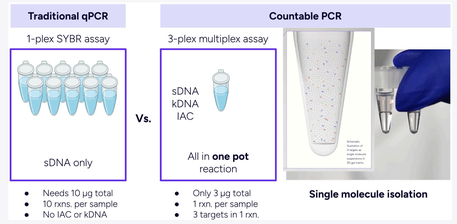


Figure 4. **Finding the needle.** Traditional methods require replicate reactions to detect a rare event in a bulk reaction. Countable PCR divides each sample into millions of isolated reactions, each one counted independently, for true single molecule detection of parasite DNA, all in one tube.

Evaluating assay performance in samples from infected subjects.

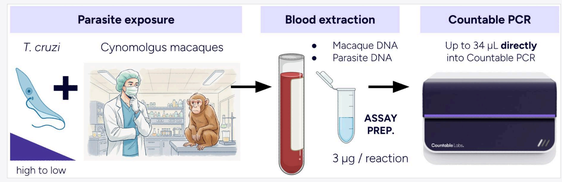


Figure 5. This study evaluated the detection of *T. cruzi* burden in a cohort of 45 infected macaques, which were exposed to varying loads of parasite (from high to low), to explore the range and sensitivity of a traditional qPCR assay and the Countable PCR assay.

Results

Countable PCR exhibited a sensitivity comparable to qPCR deep sampling for the sDNA target but using only 1/10th as much DNA.

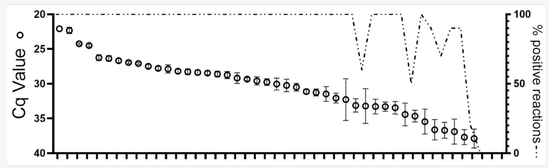


Figure 6. **Low abundance samples need more replicates in detection.** Samples that amplify above Cq 33 are detected in all replicate reactions. Below this value, where low abundance samples exist, not all reactions are positive even though they are (but at low infectivity). Detection can take 100+ replicates just to find a single positive.

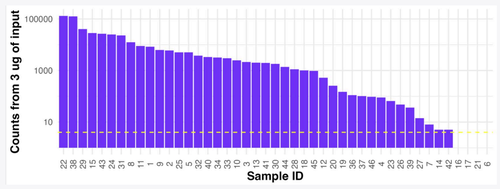
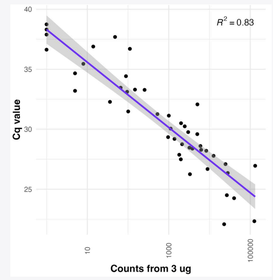


Figure 7. **Do more with less.** Using the same samples as Figure 6 but with 1/10th as much DNA and run as a 3-plex reaction, each sample required only 1 reaction to recapitulate the same sDNA detection sensitivity as seen by qPCR. The limit of detection (LoD) for the Countable PCR sDNA assay was 4 counts (yellow line). The dynamic range of Countable PCR captured the whole range of parasitic DNA.

Results, cont.

A strong correlation exists between Countable PCR and qPCR.



Comparing between:
3 µg in 1 rxn (Countable PCR)
10 µg over 10 rxns (qPCR).

Figure 8. Parasite detection in qPCR (Cq values) vs. Countable PCR (as counts) for sDNA target. Data points cover a range of *T. cruzi infected* samples, plotted to compare qPCR cycle thresholds to Countable PCR counts.

Detection sensitivity in qPCR is recapitulated in Countable PCR

kDNA represents an even more sensitive detection target.

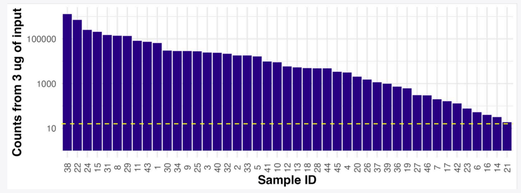


Figure 9. **Targeting a different parasite repeat element enhances detection.** Adding kDNA as part of the 3-plex assay greatly enhanced detection sensitivity for the 45 macaque samples. Some samples previous called "negative" by sDNA with qPCR or Countable PCR were detected with the kDNA target in Countable PCR. The limit of detection (LoD) for the Countable PCR kDNA assay was 16 counts (yellow dashed line). Abundance of parasite kDNA ranged from over 1 million counts/sample down to above the LoD, at ~20 counts/sample

Countable PCR detected positive *T. cruzi* cases using a single reaction, as compared to qPCR deep sampling requiring >100 reactions

Table 1. Samples with the highest Cq that were not 100% positive for all qPCR reactions are listed side-by-side with Countable PCR counts for parasite targets. Samples that took in some cases over 100 qPCR reactions to be called positive was captured in a single Countable PCR run for kDNA.

Sample ID	Average Cq	# qPCR rxns.	% pos. qPCR rxns	# pos. qPCR rxns	# Countable PCR rxns.	sDNA counts	kDNA counts
21	38.74	130	13%	17	1	1	19
16	38.31	154	27%	42	1	1	40
6	37.89	10	20%	2	1	1	53
26	37.69	10	90%	9	1	48	1501
27	36.89	10	90%	9	1	14	302
36	36.70	10	70%	7	1	111	734
17	36.63	10	90%	9	1	1	164

Conclusion

The *T. cruzi* Countable PCR assay compresses the sensitivity and dynamic range of hundreds of qPCR reactions into a single multiplexed reaction. This simple to implement workflow unlocks practical applications in early diagnosis, clinical trial assessments, post-treatment monitoring, and immune-response studies

- Detection of parasitic load, especially for low abundance infection, benefits from single molecule isolation of repeat elements
- A million-fold dynamic counting range allows detection of targets that can vary from low to high abundance across samples without the need to dilute abundant samples
- These key features of Countable PCR means samples can be run in a single reaction without any loss in detection capability

Learn more about how Countable PCR transforms rare target detection.