Countable Labs.

Multiplex detection of *BCR-ABL1* fusion transcripts in a streamlined single-tube workflow using PCR-based single molecule counting.

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Abstract

Accurate quantification of *BCR-ABL1* fusion transcripts is essential for monitoring minimal residual disease (MRD) in chronic myeloid leukemia (CML), where discrimination among clinically relevant isoforms (p210, p190, and p230) informs diagnosis, prognosis, and treatment monitoring. Conventional approaches typically use a two-step workflow consisting of reverse transcription (RT) followed by PCR amplification by either qPCR or digital PCR (dPCR). Although dPCR enables absolute quantification without standard curves, its limited dynamic range and multi-step workflow can complicate streamlined, multiplexed isoform detection.

Here, we developed a one-step multiplex RNA assay for direct detection and molecular counting of p210, p190, and p230 isoforms in a single tube, even in the presence of abundant wild-type (wt) *ABL1* transcripts. The full workflow, including RT, PCR amplification, and molecular counting, is performed without sample transfer, reducing hands-on time and minimizing contamination risk. Using 3D light-sheet microscopy, molecules are directly counted across the entire 50 µL reaction volume, eliminating dead volume and avoiding reliance on partition-based statistics.

To evaluate assay performance, *BCR-ABL1* RNA representing each isoform was serially diluted into wt *ABL1* background at varying ratios. Across dilutions, wt *ABL1* counts remained stable at high numbers, while *BCR-ABL1* counts decreased proportionally with dilution. These results demonstrate sensitive and specific detection of *BCR-ABL1* RNA isoforms across a 6-log dynamic range with low variability, enabling quantification of both abundant and rare transcripts within a single reaction.

In conclusion, this single-tube approach provides a streamlined alternative for sensitive detection of all *BCR-ABL1* isoforms in one multiplexed reaction, with potential applications in clinical assessment and therapeutic decision-making in CML.

Background

The *BCR-ABL1* fusion gene (Philadelphia chromosome) is a genetic marker of CML and is also found in a subset of acute lymphoblastic leukemia (ALL). The fusion breakpoint determines the transcript isoform, which has important implications for diagnosis, assay selection, and disease monitoring. The major CML-associated isoforms are e13a2 and e14a2 (p210), whereas e1a2 (p190) is more commonly associated with Ph+ ALL, and e19a2 (p230) is rare. Because different isoforms are linked to distinct disease settings and require different detection assays, accurate isoform identification is essential to avoid false-negative results and ensure appropriate molecular monitoring.

In this study, we designed a 5-color assay to distinguish four *BCR-ABL1* isoforms (e14a2, e13a2, e1a2, and e19a2), along with detection of wt *ABL1*, enabling accurate quantification across a wide dynamic range of *BCR-ABL1* transcript levels. The assay design schematic is shown in **Figure 1**. Each *BCR-ABL1* isoform is labeled using single- or dual-color fluorescence combinations to enhance specificity and enable modular detection of RNA fusions.

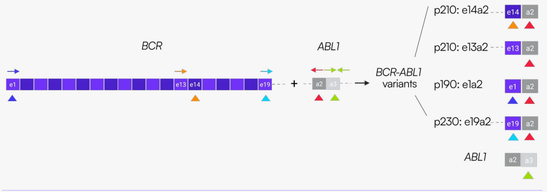


Figure 1. Schematic of multiplex detection of *BCR-ABL1* isoforms. Multiplex assay design showing primer (arrows) and probe with distinct dye (triangles) across *BCR* exons and *ABL1*, enabling isoform-specific detection via combinatorial fluorescent signals.

Methods

Streamlined RNA fusion quantification by single-molecule RNA counting.

- **True single-molecule counting:** ~30 million compartments are generated in a single PCR tube, forming a clear gel-like matrix that isolates up to one million RNA molecules for independent amplification without co-occupancy.
- **Streamlined RNA workflow:** Single-tube compartmentalization, RT, PCR, and counting—no transfers, reduced contamination. Using 3D light-sheet microscopy, molecules are directly quantified by imaging the entire 50 µL reaction volume.
- **Isoform identification using linked multi-dye approach:** By leveraging the static nature of the matrix, the same compartments can be imaged across multiple fluorescent channels to assess co-existence of signals within individual compartments. The patterns of multiple fluorescent signals is then used to identify specific *BCR-ABL1* isoform, increasing specificity.

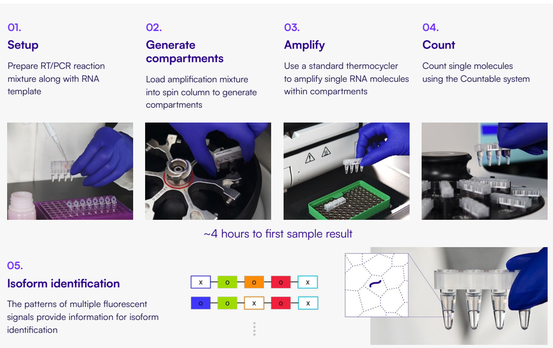


Figure 2. Streamlined RNA fusion quantification by single-molecule counting with Countable PCR. In Countable RT-PCR, target RNA molecules are partitioned into ~30 million compartments within an optically clear matrix. RT, PCR amplification, and counting all occur within a single tube. The system employs high-resolution 3D light-sheet imaging to enable rapid and precise quantification. In addition, linkage analysis enables identification of specific isoforms and automated reporting of %IS.

Countable PCR enables single reaction per sample → no standard curves, multiplex RNA workflow.

Figure 3A shows a representative qPCR plate setup for 8 samples, requiring 52 reactions (including calibrators) and lacking multiplex capability. In contrast, Countable PCR (**Figure 3B**) uses a single reaction per sample for isoform identification, with no limits on multiplexing or dynamic range, and provides automated %IS without calibration factors.

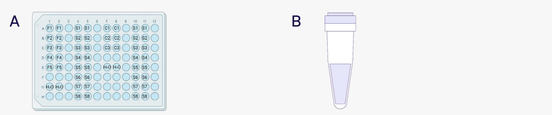
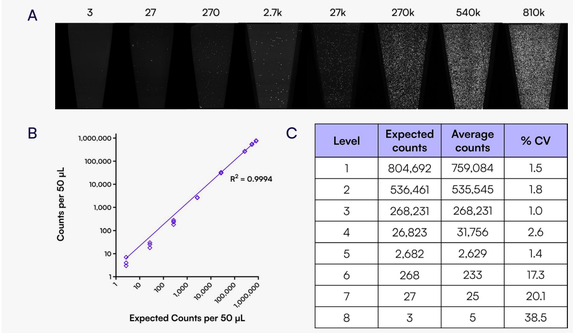


Figure 3. Comparison of sample setup: qPCR (A) vs Countable PCR (B).

Results

One-step Countable RT-PCR delivers direct RNA quantification over a 6-log linear dynamic range.

ABL1 counts were first quantified using a single-plex assay with titrated HL60 RNA in the Countable system. 1 µg of HL60 RNA generated around 270,000 *ABL1* counts and up to 3 µg of HL60 RNA was loaded. As shown in **Figure 4**, Countable RT-PCR system produced linear counts across a 6-log dynamic range (R^2 = 0.9994), with %CV < 3% at inputs above 1,000 copies. Representative light sheet images at different RNA levels and their corresponding counts are shown below.



Level	Expected counts	Average counts	% CV
1	804,692	759,084	1.5
2	536,461	535,545	1.8
3	268,231	268,231	1.0
4	26,823	31,756	2.6
5	2,682	2,629	1.4
6	268	233	17.3
7	27	25	20.1
8	3	5	38.5

Figure 4. Linear dynamic range up to 6-log dynamic range. Single-plex assay for *ABL1* was tested with titration of HL60 RNA (N=4). (A) Light sheet images at different RNA target levels are shown. The corresponding counts for each level are plotted in (B), with a summary provided in (C).

Multiplexed isoform quantification confirmed in a single tube.

RNA carrying each *BCR-ABL1* variant from cell lines was used as the template. RNA template was incubated with its corresponding single-assay or multiplexed condition (e14a2/e13a2/e1a2/*ABL1* assays combined). The average counts from single-plex and multi-plex assays were comparable, demonstrating assay robustness in the multiplex condition.

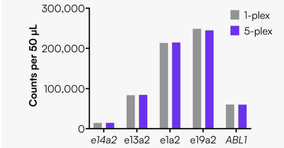


Figure 5. Multiplexing Capability of the *BCR-ABL1* Assay. Comparable counts were shown for each *BCR-ABL1* variant RNA template under both single-plex and multi-plex assay conditions.

The broad dynamic range enables detection of rare *BCR-ABL1* events in the presence of abundant *ABL1*, making it suitable for MRD monitoring.

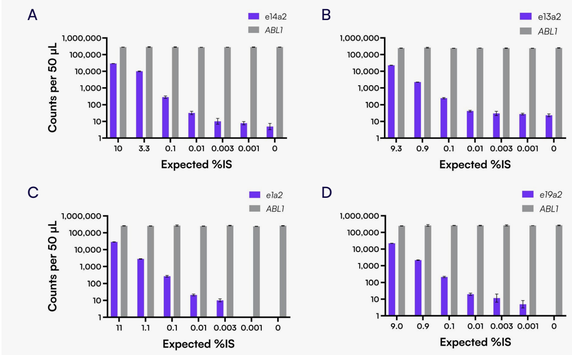


Figure 6. Quantification of different ratios of *BCR-ABL1* variants to wt *ABL1*. RNA containing e14a2 (A), e13a2 (B), e1a2 (C), and e19a2 (D) was diluted into wt *ABL1* to generate varying %IS. wt *ABL1* counts remained constant, while *BCR-ABL1* counts decreased proportionally to low tens of molecules (N = 4), enabling single-tube discrimination of all isoforms.

Results, cont.

RNA containing the e14a2, e13a2, e1a2 and e19a2 isoforms was diluted into a background of wt *ABL1* RNA to generate varying *BCR-ABL1* to wt *ABL1* ratios. While wt *ABL1* counts remained constant, the counts of *BCR-ABL1* variants decreased proportionally with dilution.

Conventional dPCR platforms are limited at high *ABL1* input due to partition oversaturation, constraining simultaneous detection of both high- and low-abundance targets. The broad dynamic range of the Countable RT-PCR system enables simultaneous detection of both low- and high-abundance RNA targets within a single tube.

Moreover, our assay can distinguish *BCR-ABL1* isoforms in a single tube, eliminating the need to run multiple single-plex assays for each isoform.

Detecting RNA fusion variants as low as 0.003% IS.

When measured %IS values were compared with the expected %IS (calculated based on the counts at the highest level for each target and further dilutions for subsequent levels), results from the highest level (~10%) down to 0.003% showed close agreement.

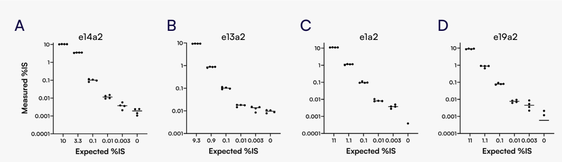
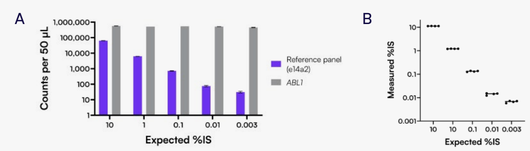


Figure 7. Comparison of Expected vs Measured %IS. Measured %IS was plotted against expected %IS for each *BCR-ABL1* variant: (A) e14a2, (B) e13a2, (C) e1a2, and (D) e19a2 (N=4). The measured %IS matched the expected values across tested levels.

Results of *BCR-ABL1* Countable RT-PCR assay match the %IS of reference panel.

The *BCR-ABL1* assay was evaluated using an AcroMetrix reference panel with e14a2 to wt *ABL1* ratios of 10%, 1%, 0.1%, 0.01%, and 0.0032%. The measured results closely matched the expected values across all levels, with low %CV. The assay reliably detected *BCR-ABL1* levels down to 0.0032%. Detection at this level is clinically significant, as it informs therapeutic decisions, including eligibility for tyrosine kinase inhibitor discontinuation in CML patients.



C

Expected %IS	Measured %IS		Counts of e14a2		Counts of ABL1	
	Value	%CV	Value	%CV	Value	%CV
10	9.8	2.4	68,604	1.7	702,195	1.2
1	1.0	2.1	5,490	2.0	575,003	0.6
0.1	0.1	8.6	563	7.8	560,625	0.8
0.01	0.01	13.0	51	12.5	550,339	1.0
0.0032	0.002	37.8	13	38.9	551,783	1.8

Figure 8. Characterization of the *BCR-ABL1* assay using AcroMetrix reference panel. The assay was evaluated using a reference panel with known *BCR-ABL1* to wt *ABL1* ratios. Counts of e14a2 and *ABL1* (A), the corresponding %IS values (B), and a summary table (C) are shown (N=4).

Conclusion

Countable PCR enables multiplex detection of all four *BCR-ABL1* isoforms (e14a2, e13a2, e1a2, and e19a2) in a streamlined single-tube workflow, eliminating the need for separate RT steps and standard curve based quantification.

The assay demonstrates a 6-log linear dynamic range, supporting accurate quantification from high tumor burden at diagnosis through deep MRD monitoring within a single reaction.

A sensitivity of 0.0032% IS validated against a reference panel, meeting the MR4.5 threshold relevant for treatment discontinuation decisions in CML.

Overall, these results position Countable PCR as a streamlined alternative to conventional multi-step qPCR workflows for comprehensive *BCR-ABL1* monitoring.

Learn more about how Countable PCR transforms multiplex *BCR-ABL1* fusion detection from direct RNA input.