Countable Labs.

A high-sensitivity PCR assay for multiplexed detection via single-molecule counting of *BRAF* mutations from low-input liquid biopsy samples.

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Abstract

BRAF is a critical gene of interest due to its implication in many cancers, including thyroid, melanoma, and colorectal cancer. Specifically, clinically-relevant mutations V600E, V600K, and V600R have been identified as the most common oncogenic mutations, and the presence of single- or double-point mutations in patients are important factors in therapeutic decision-making.

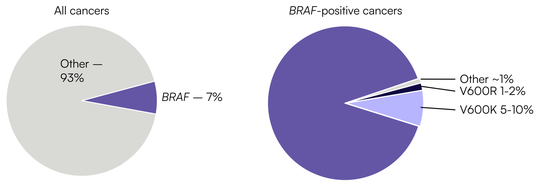
Accurate detection of *BRAF* mutations in liquid biopsy samples remains challenging due to the limited sample available for analysis and the difficulty of distinguishing closely related variants. Conventional qPCR and digital PCR (dPCR) approaches are constrained by low input tolerance, limited dynamic range, and poor scalability for multiplexed mutation detection. Due to these technical limitations, these approaches are limited to VAF% (variable allele frequency) of about 1-5% for qPCR and 0.1% for dPCR, but researchers demand more sensitive and faster VAF% determination as therapeutic treatments evolve.

Here, we describe a one-tube, 4-plex PCR assay capable of distinguishing and quantifying *BRAF* mutations with high sensitivity via single-molecule counting. With the method introduced here, combined with an optional pre-amplification step, the assay achieves a 6-log dynamic range, enabling direct comparison of WT and low abundance mutants. Furthermore, with no dead volume, it was possible to directly amplify low-abundance targets from 35 µL of input sample, effectively increasing the sensitivity of the methodology.

These abilities allowed us to improve the limit of detection (LoD) and confidence in the results, demonstrating detection of rare variants down to ~0.05% VAF. The ability to measure all common *BRAF* mutations in one tube preserves precious samples.

Background

BRAF cancer treatment begins with accurate detection.



- Early diagnosis guides treatment decisions and monitors treatment
- Targeted therapies for these mutations are generally the same
- V600 mutations are sensitive to the same BRAF and MEK inhibitors
- Current V600 screening kits lack sensitivity and have low throughput

Countable PCR workflow for detecting rare molecule targets in cfDNA.

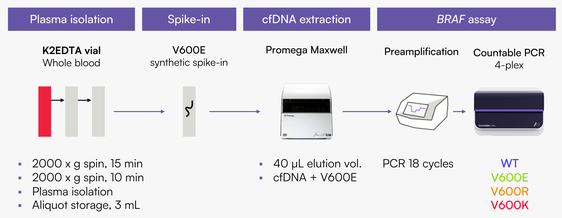


Figure 1. Rare molecule detection starts with (1) plasma isolation from whole blood. (2) Synthetic mutant BRAF spike-in is done at a chosen %VAF. (3) cfDNA extraction with Promega Maxwell® CSC prepares samples for (4) preamplification in a benchtop thermal cycler and *BRAF* multiplex assay run on Countable PCR.

The Countable Labs 4-Plex *BRAF* assay uses a half-nested PCR preamplification step to improve the sensitivity of detection.

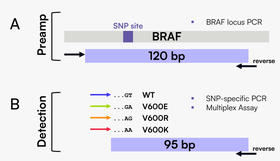


Figure 2. Schematic design of Countable PCR BRAF assay. Half-nested PCR primer sets were designed to amplify the BRAF locus from 25 ng of cfDNA

Half-nested primers were designed to (**Figure 2, A**) amplify the *BRAF* locus (a preamp) in a bulk PCR reaction, independent of SNP mutation status. Once amplified (**Figure 2, B**), samples were taken directly into Countable PCR for mutation specific detection with a 4-plex *BRAF* multiplex assay. SNP primers were designed using Universal Multiplexing (UM) chemistry.

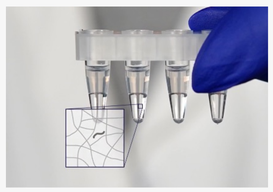


Figure 3. Single molecule isolation happens into a picoliter-sized compartment created during the matrix preparation of Countable PCR workflow.

Single molecule isolation and counting with Countable PCR.

Countable PCR creates ~30 million isolated compartments through centrifugation to enable true single-molecule quantitation (**Figure 3**). Each target molecule is isolated and amplified independently in its own compartment, eliminating amplification bias for multiplex assays, and enabling direct counting .

Results

Counts were determined with Countable Control Software on the Countable System and sample quality indicators were reported via the Countable Control Analysis Report.

Assay performance is within specifications for single and multi-targets.

Combinations of synthetic mutant spike-ins were tested for specificity in a 4-plex assay (**Table 1**). Combinations were tested either directly in Countable PCR (Native, ~6,000 counts) or after 18-cycles of PCR amplification (Preamp) that increased the total number of molecules in each reaction to ~570,000 counts.

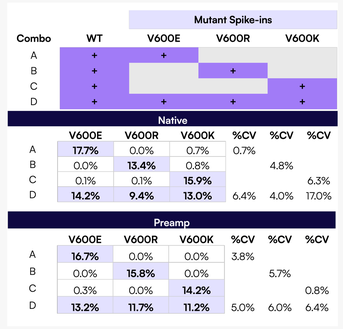


Table 1. *BRAF* assay performance for the native condition was as expected. The correct on-target %VAF had only minimal off-target signal (<0.8%).

Preamp followed this trend, with the correct on-target %VAF, tight %CV, and <0.3% off-target signal.

Single target VAF was expected to be ~15%; for three targets at once (seen in combo D), expected 12% for each target.

These data shows that amplification does not bias counting.

A dilution series of cfDNA reached an ultra low %VAF for the BRAF V600E mutation

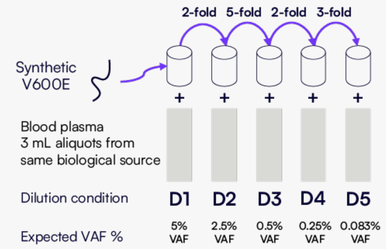


Figure 4. Dilution scheme for 4-plex BRAF determination assay. Extracted cfDNA from human plasma was used to determine the yield and quality of each biological replicate to determine how much synthetic V600E to spike into the remaining aliquots to create a %VAF series across five points with increasing fold dilution, starting at 5% expected for sample D1.

Lowering the limit of detection for V600E with Countable PCR

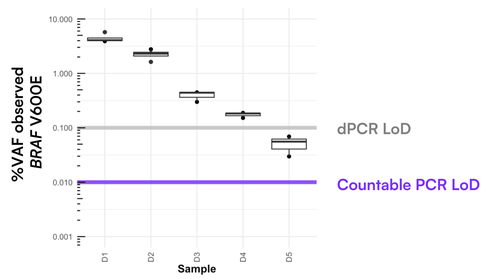


Figure 5. *BRAF* V600E detection reached 0.05% VAF in cfDNA samples done as biological replicates. Countable PCR surpassed current detection limits.

The broad dynamic counting range of Countable PCR allowed detection of >700,000 WT molecules against ~400 V600E mutant counts in a single reaction (**Figure 5, D5**). The determined VAFs were all above an experimentally determined limit of detection (purple line) in contrast to dPCR platforms (gray line).

Results, cont.

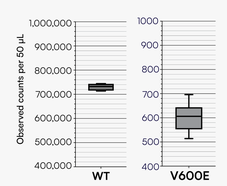
Countable PCR achieves 0.05% VAF for V600E in cfDNA.

Table 2. Summary of the cfDNA V600E spike-in experiments. Biological replicates were tested over a wide range of mutant VAFs down to 60-fold from starting VAF of ~5%. For the highest dilution (D5), more than 700,000 molecules were loaded into a single reaction to count rare target above the LoD of 70 counts for V600E.

Sample	Dilution	Average counts		Average %VAF	%CV	Replicates
		WT	V600E			
D1	1-fold	176,391	8,782	4.45%	19.4%	N=4
D2	2-fold	127,337	2,772	2.25%	21.1%	N=4
D3	10-fold	217,966	850	0.32%	21.3%	N=3
D4	20-fold	171,698	293	0.17%	11.3%	N=3
D5	60-fold	747,848	397	**0.05%**	38.8%	N=3

Technical reproducibility is consistent for low %VAF sample.

Continuing from the V600E %VAF dilution experiment, the sample with the lowest VAF was re-run as technical replicates to determine the consistency of absolute counts for the assay targets in the context of extracted cfDNA (**Figure 6, Table 3**).



Sample ID	WT counts	V600E counts	%VAF
A1D5_rep1	743,320	514	0.07%
A1D5_rep2	744,129	620	0.08%
A1D5_rep3	715,268	644	0.09%
A1D5_rep4	732,423	564	0.08%
A1D5_rep5	736,731	696	0.09%
A1D5_rep6	713,717	552	0.08%
A1D5_rep7	720,248	606	0.08%
Average	729,405	599	0.08%
Standard Dev.	11,970	57	0.01%
%CV	**1.64%**	**9.48%**	**9.61%**

Figure 6 and Table 3. Counts for 7 technical replicates run on two different days, visualized. A very low %CV (<2% and <10%) was observed for individual assay targets and the final %VAF calculation.

Conclusion

The Countable PCR single tube BRAF 4-plex assay detects below 0.05% VAF for the V600E mutation in human cfDNA samples.

These results describe a general multiplexing PCR methodology for highly sensitive detection of rare variants from low-abundance DNA samples, applicable for many clinically-relevant examples. This approach has the potential to address key limitations in current detection workflows involving low-input material such as cell-free DNA, fine-needle aspirates, small tissue biopsies, and FFPE samples. For this assay:

- We developed a 4-plex assay for *BRAF* mutations and wild-type, as an all-in-one reaction
- We measured from ~400 to over 700,000 targets in the same reaction, which is possible due to the broad dynamic counting range of Countable PCR
- We pushed the VAF of *BRAF* V600E down to 0.05% with potential for even greater sensitivity

Ultimately, this technology supports more sensitive and efficient detection of clinically-relevant mutations across diverse oncology applications.

Learn more about how Countable PCR transforms sensitive target detection.

Special thanks to Promega for the use of the Maxwell CSC extraction device.