Countable Labs. Promega

Development of a single-tube, 8-color PCR assay for quantifying a *KRAS* mutation panel using true single-molecule counting.

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Abstract

KRAS mutations are among the most common drivers of malignant tumors in lung, colorectal, and pancreatic cancers. Identifying specific mutations in solid tumors guides treatment selection, while quantifying mutant-to-wild-type alleles in liquid biopsies helps assess therapeutic response and recurrence risk. Accurate mutation profiling is essential for developing KRAS-targeted therapies.

However, standard qPCR assays detect mutations only at ~1% mutant allele frequency (MAF) and require multiple reactions, increasing cost and reducing scalability. Digital PCR (dPCR) improves the sensitivity to ~0.2% MAF but often groups different targets into shared channels, limiting specificity. For low-abundance, limited-input samples such as cell-free DNA (cfDNA), a more sensitive and efficient assay is urgently needed that addresses both sensitivity and specificity challenges.

Here, we present a robust PCR-based method for simultaneous quantification of multiple *KRAS* mutations in a single reaction.

Background

- 99% of known *KRAS* mutations are covered by 7 exclusive single base pair mutations
- Some *KRAS* mutations can be drugged (G12C, G12V, G12D), while other mutations have yet to be treatable
- Knowing a specific *KRAS* mutation helps guide treatment options
- Countable PCR overcomes the multiplexing limitations of qPCR and dPCR, enabling precise quantitation of gene panels in a single reaction, providing a practical solution for biomarker validation post NGS studies.

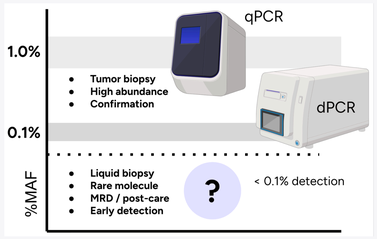

Figure 1. Rare target discovery is limited by detection sensitivity. *KRAS* assays that rely on qPCR and dPCR have limited sensitivity, reducing their effectiveness in liquid biopsy (cfDNA) screening, post-treatment minimal residual disease (MRD) screening, and early detection testing.

Method

Countable PCR 8-color *KRAS* assay uses a pre-amplification step to improve the sensitivity of detection.

We utilized Countable PCR, which enables direct counting of single molecules across a broad dynamic range, to develop an 8-color assay that differentiates and quantifies both common and rare *KRAS* mutations in a single reaction, with higher precision and sensitivity than other platforms. The inclusion of a SNP-agnostic pre-amplification step accurately quantifies abundant wild-type and low-frequency mutant allele without loss of accuracy. Each *KRAS* mutation (G12C, G12V, G13D, G12D, G12A, G12S, G12R), along with the wild-type allele, is represented by one of eight unique optical signatures.

Method, cont.

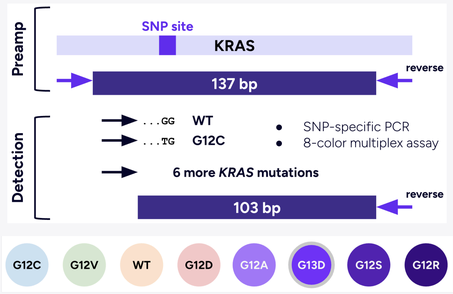

Figure 2. 8-color assay design. Half-nested PCR primer sets were designed to first amplify the *KRAS* locus (preamp) independent of SNP status in a bulk reaction (purple arrows). Once amplified, samples were taken directly into Countable PCR for SNP-specific detection using an 8-color multiplex assay (the black arrows with the purple reverse). The *KRAS* targets are listed by amino acid mutation within a colorized circle (optical signature).

A streamlined workflow with the Promega Maxwell® CSC makes cfDNA processing easier that is compatible with Countable PCR.

Total cfDNA was extracted from human blood plasma using a magnetic bead-based automated system (Rapid ccfDNA Kit on the Maxwell® CSC system) to ensure consistent recovery from low-input material.

Single molecule isolation and counting with Countable PCR.

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

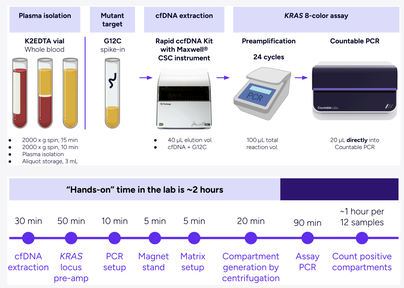

Figure 3. A complete workflow. The workflow begins with plasma isolation from whole blood, synthetic DNA spike-in at a chosen %MAF, cfDNA extraction using the Rapid ccfDNA Kit on the Maxwell® CSC system, *KRAS* target pre-amplification to boost sensitivity, and the *KRAS* 8-plex assay run on Countable PCR.

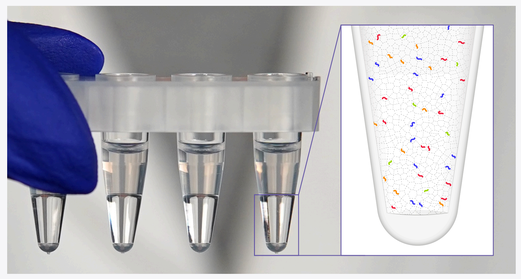

Figure 4. Schematic illustrating the single-molecule isolation of 4 targets into ~30 million picoliter-sized compartments.

Results

Each of the 8-color targets in the assay are detected by a unique optical signature.

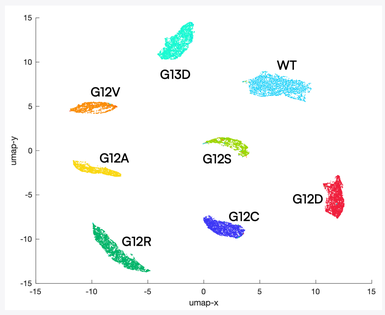

Figure 5. Each target color is distinctly identified. The optical signature in each positive compartment of the Countable matrix is projected onto a 2-dimensional representation using Uniform Manifold Approximation and Projection (the UMAP) analysis. Compartments with similar signatures are placed into the same cluster and are called to a type based on a training set of single-targets that define the map. As shown, each target in the 8-color assay is clearly separated, allowing for determination of counts of each target based on the the number of compartments assigned to a cluster.

Results, cont.

8-color assay achieves the expected performance in counting and specificity against different mutants.

KRAS mutant-specific synthetic DNA templates were tested for on-target specificity as a 1-color or an 8-color assay. To show no counting bias, counts from a 1-color Countable PCR for each mutation should match the counts for that same mutation in the 8-color, and the NTC counts for all targets should be as low as possible relative to real counts.

Table 1. For each mutation tested using a single template in either a 1-color or 8-color PCR reaction, counts are shown per reaction. Along the diagonal (purple boxes), we expect to see counts consistent with the 1-color reaction by *KRAS* mutation type.

1-Plex Counts	G12C	G12S	G12V	G12D	G12A	G12R	G13D	WT
	3,097	1,769	1,993	3,363	2,107	3,060	3,446	5,712

8-Plex Counts	G12C	G12S	G12V	G12D	G12A	G12R	G13D	WT
G12C	3,149	7	1	0	6	5	0	3
G12S	1	1,792	1	2	0	0	1	19
G12V	0	6	2,059	1	8	1	0	4
G12D	0	2	5	3,701	13	0	0	18
G12A	0	1	4	1	2,115	1	1	1
G12R	1	1	0	0	0	3,922	1	5
G13D	0	1	0	2	0	1	4,016	22
WT	4	4	1	4	0	2	105	5,325

NTC Counts	G12C	G12S	G12V	G12D	G12A	G12R	G13D	WT
	12	3	3	3	21	5	7	10

Determining sensitivity of *KRAS* detection in extracted samples.

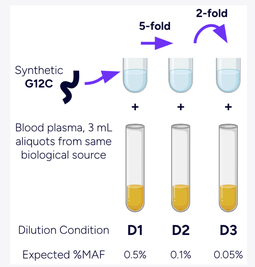

Figure 6. Dilution scheme. Total cfDNA was extracted from 3 mL of human plasma using the Rapid ccfDNA Kit on the Promega Maxwell® CSC system. Aliquots were spiked-in with dilutions from a synthetic *KRAS* G12C template to create an estimated %MAF over three points of diminishing abundance, starting from an expected MAF of 0.5% in sample D1.

Countable PCR confidently detects 0.08% MAF for G12C in cfDNA.

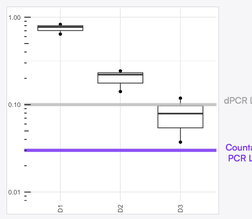

Figure 7. Pre-amplification of cfDNA boosted the number of mutant targets above background. Support of a broad counting range allowed the Countable PCR *KRAS* assay to detect rare targets better than existing methods (purple vs. gray LoD line).

The broad counting range of Countable PCR allowed the detection of >140,000 WT *KRAS* molecules (Figure 7 and Table 2) against ~113 G12C counts in a single reaction, an average MAF of 0.08% and above the experimentally determined limit of detection (purple line, an average of 44 counts, N=8), in contrast to dPCR assays (gray line) that cannot reliably detect below 0.1% MAF.

Table 2. Molecule counts per reaction for each dilution, shown as average value of technical replicates. Average counts are for wild-type and G12C mutant after pre-amplification from 25 ng of total cfDNA. The expected copy input of G12C was ~5 copies per reaction into pre-amplification.

		WT	G12C			
Sample	Dilution	Ave. counts	Ave. counts	Ave. %MAF	%CV	Replicates
D1	1-fold	143,030	1,072	0.75%	12.8%	N=3
D2	5-fold	109,247	216	0.20%	26.5%	N=3
D3	10-fold	147,206	113	0.08%	51.9%	N=3
Blank	-	-	18	-	-	N=8

Conclusion

The Countable PCR single tube *KRAS* 8-color assay can detect 0.08% MAF for the G12C mutation in human cfDNA samples.

We unambiguously identified seven *KRAS* mutants in a single reaction. When coupled with a pre-amplification step, the assay showed detection sensitivity to 0.08% for the G12C mutation, approximately one order of magnitude more sensitive than published assays. Results were reproducible across technical replicates and were achieved with minimal optimization.

- We developed an 8-color assay for *KRAS* mutations and wild-type, all in one reaction
- Using pre-amplification to boost target counts, we measured over 140,000 targets in the same reaction, demonstrating how a broad dynamic counting range supports sensitive target detection
- Countable PCR is compatible with the Rapid ccfDNA Kit on the Maxwell® CSC system for cfDNA extraction

Using *KRAS* cancer mutations as an example, we showed that Countable PCR enables profiling of multiple targets in a single experiment with high sensitivity and reduced time for optimization. With straightforward and sensitive 8-color multiplexing, this approach opens new avenues for research and diagnostic panels targeting other cancers.

Learn more about how Countable PCR transforms rare target detection.