App Note

Countable Labs.

Straightforward pre-amplification protocols in Countable PCR improve sensitivity of your rare event detection

Get actual counts of your rare and abundant molecules at the same time — for precise VAF% determination without concerns about detector saturation or lost sensitivity.

Introduction

PCR is a critical tool for detection of rare markers that indicate disease[1]. In highly sensitive applications such as oncology and infectious disease, target DNA is often derived from patient samples like cell-free DNA (cfDNA) obtained via liquid biopsy[2]. These samples contain very low amounts of DNA, and reliably identifying and quantifying the amount of mutant or foreign DNA compared to WT directly from them is challenging.

At present, digital PCR (dPCR) is widely used for rare molecule detection, as it enables a relatively sensitive and scalable approach to calculate the Variable Allele Frequency (VAF), a common unit of measurement used in oncology. However, when working with highly dilute inputs like liquid biopsy samples, the presence of rare molecules is often so low that there is a lack of statistical confidence when determining their presence. To combat this, researchers turn to pre-amplification protocols, which use an extra step prior to the quantification approach to boost the amount of DNA in the sample, while maintaining the ratio between mutant and WT or foreign vs native DNA[3].

With pre-amplification protocols, dPCR has notable drawbacks that limit its effectiveness as a method for rare molecule detection:

- dPCR relies on Poisson statistics to estimate molecule number, and detection using current dPCR platforms is limited to a 5-log range due to saturation at the high end. Detection of high-frequency and low-frequency events from the same sample is thus extremely difficult to perform with use of pre-amplification, due to saturation of signal from high-count targets. Thus, parallel experiments are run instead, which is inefficient and costly.
- Microfluidics-based dPCR also suffers from low input capacity, limiting the analyzable volume per reaction. This necessitates splitting target sample across multiple wells, and therefore running multiple reactions to ensure no rare molecule is missed from the pre-amplified sample.

With Countable PCR, we introduce the ability to enable detection of rare variants down to 0.003% VAF with minimal optimization and without special reagents. Here, we show an approach for expanding the search for rare molecules using a pre-amplification protocol, without requiring multiple experiments to reliably determine %VAF. With Countable PCR's broad dynamic range, it is straightforward to compare WT and mutant in the same reaction, in a single tube, without dilution or parallel reactions[4]. The result is very low %VAF sensitivity with good statistical confidence in a single reaction.

Materials

Reagents and consumables

Component	Vendor	Catalog No.
Q5 High-Fidelity 2X Master Mix	NEB	M0492S
4X Countable PCR Mix Kit	Countable Labs	KT0004
Countable Matrix Consumables Kit	Countable Labs	KT0001
Countable Matrix Reagents Kit	Countable Labs	KT0003

Experimental setup

Sample dilution

Four dilutions were prepared from a synthetic *JAK2 V617F* mutation sample with WT human genomic DNA. Dilutions started at 30% VAF and diluted 1:10 until 0.03% VAF was achieved.

Direct sample input for % VAF determination (no pre-amplification)

1. We prepared a dilution series of the *JAK2 V617F* mutation in the background of WT human genomic DNA.
2. We input 80 ng of each diluted sample into a Countable PCR reaction according to the Countable PCR Reaction Preparation Guide.
3. Prepared samples were then loaded into the Countable Instrument for imaging and automatic counting according to the Countable PCR Setup Guide.

12-cycle pre-amplification step for % VAF determination

1. For each sample in the dilution series, we prepared the following amplification mix:

Component	Per 50 µL reaction	Catalog No.
Q5 High-Fidelity 2X Master Mix	12.5 µL	1X
10 µM Forward Primer	2.5 µL	0.5 µM
10 µM Reverse Primer	2.5 µL	0.5 µM
Template DNA (from each dilution step)	Variable	200k molecules (ca. 600 ng)
Nuclease-free Water	To 50 µL	—

2. Samples underwent thermal cycling with the following annealing temperatures in a BioRad C1000 Touch Thermal Cycler (sample reaction volume = 50 µL; heated lid temperature = 105°)

Cycle	Step	Temperature (°C)	Time
1	Initial denaturation	98	30 sec
12	Denaturation	98	10 sec
	Annealing	65	30 sec
	Extension	72	30 sec
1	Final extension	72	2 min
1	Store	8	∞

3. After PCR, the samples were purified using NEB Clean and Concentrator (cat# T1030).
4. A 1:40 dilution of each purified sample was prepared. At this dilution, each sample should contain ca. 250k molecules/µL.
5. For each diluted sample, 2 µL was input into a Countable PCR reaction according to Countable PCR Setup Guide.
6. Prepared samples were then loaded into the Countable Instrument for imaging and automatic counting according to Countable PCR Setup Guide.

Counting and analysis

Counts were automatically produced in V 0.6 of The Countable Control software. To report results, the Countable Instrument directly images and counts fluorescent signal from amplified single-molecule targets and reports counts of target molecules per 50 µL reaction. Hundreds of light sheet images are taken, in less than 5 minutes per tube, and then processed automatically with the Countable Control Software (V0.6). Results are reported as direct counts; representative images are exported and displayed with results.

The LoD applied to find the threshold of reporting confidence was determined from the mean NTC signal (false-positive counts) plus two standard deviations from that background average.

Results

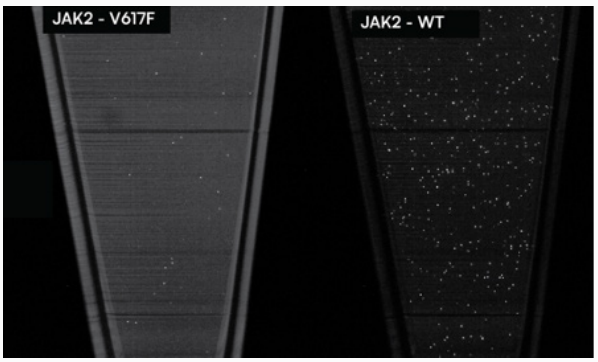
We compared the ability to determine VAF% using both a sample containing dilute amounts of *JAK2 WT* vs. oncologic mutant *JAK2 V617F*, as well as the same sample after a 12-cycle pre-amplification step. Countable PCR's matrix technology forms physically isolated compartments of single molecules, supporting unbiased amplification of multiple targets in a single tube. The matrix creates millions of individual compartments, which **enables detection across a broad dynamic range and minimizes the need to split DNA samples into multiple reactions to determine %VAF** or reach the sensitivity required for some clinical applications.

Countable PCR enables direct counting of molecules in samples containing dilute DNA

To begin, we evaluated Countable PCR's sensitivity for samples that had not undergone the pre-amplification step. Samples were prepared with both *JAK2 WT* and *JAK2 V617F* (mutant) in known ratios. These samples were dilute, containing about 23500 molecules per 50 µL, to mimic conditions from sources like liquid biopsy.

Figure 1 shows the results of the Countable PCR assay for determining %VAF for this *JAK2 WT/V617F* experiment. The blank samples gave a theoretical LOD

A.



B.

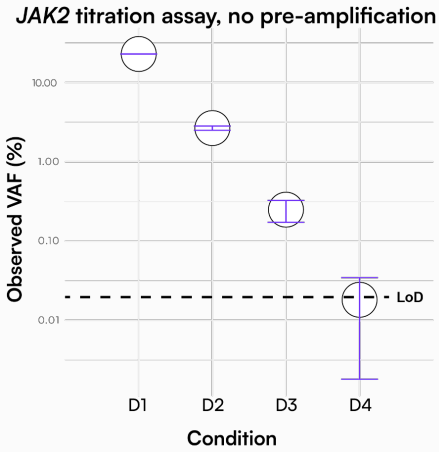
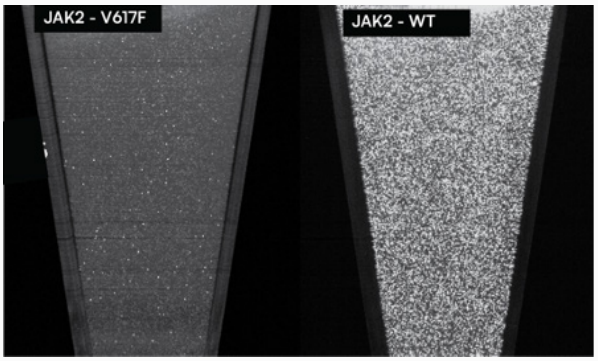


Figure 1. Results from dilute input JAK2 titration assay, non-pre-amplified sample. (A) Representative images of Countable PCR light sheet counting for non-pre-amplified sample. Images are from D2 dilution, representing 3% VAF. (B) Plotted dilution series of VAF determination from non-pre-amplified sample. Dilution 4 (D4), expected to be 0.03% VAF, was below the limit of detection, i.e., within the error from blank standards. Total counts for all samples averaged about 23500 molecules.

A.



B.

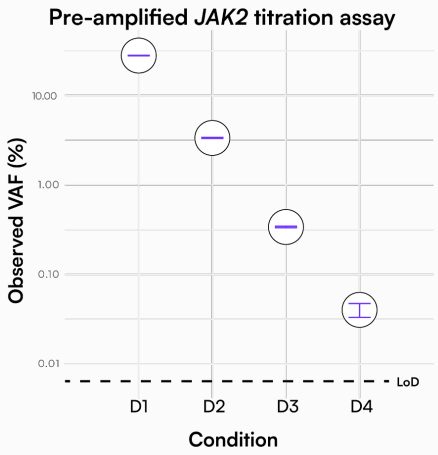


Figure 2. Results from pre-amplified JAK2 titration assay. (A) Representative images of Countable PCR light sheet counting for non-pre-amplified sample. Images are from D2 dilution, representing 3% VAF — i.e., the same dilution sample as shown in Figure 1, now pre-amplified. (B) Plotted dilution series of VAF determination from pre-amplified sample. Dilution 4 (D4), expected to be 0.03% VAF, was well above the limit of detection with the statistical confidence introduced from amplifying the total sample. Total counts for all samples averaged about 421000 molecules, well above the amount possible to determine with accuracy using dPCR.

of 0.025%, which represents 2 +/- 2 molecules per 50 µL sample. Since the average number of counts for dilution 4 (D4) is 4, it falls within the error range of the background from blank samples, and therefore is not considered statistically reliable.

Countable PCR's broad dynamic range makes %VAF with pre-amplification protocols straightforward

Pre-amplification of sample containing low amounts of DNA is a common approach for boosting statistical confidence, leveraging the law of large numbers to improve the sensitivity of rare molecule detection. With a 12-cycle pre-amplification protocol, the expected increase in amplification is 1156-fold, assuming about 1.8x amplification efficiency per cycle. The goal of pre-amplification is to increase the statistical confidence in %VAF reporting for the lowest dilution factor.

Figure 2A shows representative slices of D#, the same sample as shown in **Figure 1A** after undergoing the pre-amplification step. The total number of both WT and mutant *JAK2* has now been increased, which improves the statistical confidence in reporting the dilutions containing low amounts of *JAK2 V617F*.

Figure 2B shows the results of the titration of *JAK2* after pre-amplification. After pre-amplification of each titration sample, 1 µL of a 20-fold dilution of the final amplification product was taken and ran in Countable PCR. Total molecule counts (*JAK2 WT + V617F*) for each replicate were about 421000 — far above the dynamic range possible with dPCR. With the addition of the pre-amplification, it was now possible to accurately determine the %VAF down to the lowest dilution, 0.03% (D4). Theoretical LoD for this pre-amplified protocol is 0.003% VAF.

Table 1 shows the summary of results from the pre-amplification protocol with Countable PCR. Countable PCR gives direct counts of the number of molecules present in a 50 µL sample across a broad dynamic range. When used in conjunction with a pre-amplification protocol, it makes highly sensitive determination of %VAF

straightforward, as both rare (JAK2 V617F) and common (JAK2 WT) are counted in the same tube. There are no concerns about diluting sample to bring WT counts within the narrow dynamic range for dPCR, nor that Poisson correction will distort the counts for high-frequency events.

Conclusion

Countable PCR's broad dynamic range makes it a practical and powerful tool for rare molecule detection. Here, we demonstrate specifically how pre-amplification protocols for increasing statistical confidence in rare molecule quantification are easier thanks to the broad dynamic range possible with Countable PCR. Even with inflated amounts of abundant genes, there are no concerns about saturating the detector, as with dPCR. This means it's straightforward to compare WT versus mutant in a single pre-amplified sample, without the need to split it across multiple reactions, or perform separate dilutions for wild-type quantification. With the ability to count rare and common events in the same tube, without concerns about over-saturating the detector for Poisson correction, Countable PCR delivers lower VAF% detection with greater statistical confidence and less assay development.

As dPCR platforms approach fundamental limits for sensitivity, the demand for more sensitive rare molecule detection methods continues to grow. Countable PCR overcomes these limits by enabling highly sensitive and reliable detection of rare events for pre-amplified samples in fewer reactions. With higher analyzable volume and a greater tolerance for high-frequency events, it reduces time, cost, and effort for rare molecule detection.

Table 1. Results from both dilute and pre-amplified JAK2 titration assays.

	No pre-amplification					With pre-amplification				
	Average mt counts per 50 µL	**Average WT counts per 50 µL**	**%CV**	**n**	**Determined VAF**	**Average mt counts per 50 µL**	**Average WT counts per 50 µL**	**%CV**	**n**	**Determined VAF**
D1 - 30% VAF	6767	22702	0.14	2	23%	170045	438970	0.53	4	28%
D2 - 3% VAF	630	23010	6.2	2	2.7%	13920	401550	1.4	4	3.4%
D3 - 0.3% VAF	55	22179	31	2	0.25%	1245	365953	2.0	4	0.34%
D4 - 0.03% VAF	4	23442	90	4	0.018%	158	394965	18	8	0.040%
No Mutant	2	21685	2	4		384587	5	88	8	

References

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