Countable Labs.

# A single-molecule PCR framework bridging low-plex PCR and NGS for biomarker validation at 16-plex and beyond.

Christina Fan, Michael Porter, Jung Won Keum, Haeun Lee, Vladyslava Ratushna, Christina Cheng, Andras Saftics, Mehdi Molaei, Janice Lai, Eleen Shum

## Abstract

Next-generation sequencing (NGS) has enabled the discovery of thousands of candidate biomarkers across a wide range of diseases and traits. Translating these discoveries into practical, quantitative assays remains a major challenge. Conventional qPCR and dPCR platforms are widely used for low-plex validation, but their limited multiplexing capacity constrains scalability. Moreover, high-plex PCR assays typically require extensive optimization to balance amplification efficiencies and minimize cross-reactivity, limiting their utility for large-scale validation and clinical assay development. A quantitative platform that combines high multiplexing, digital precision, and efficient use of limited samples is needed to close this gap.

We describe a single-molecule quantitative PCR approach, Countable PCR, designed to address these challenges. The method isolates and amplifies up to one million individual DNA molecules in parallel without sample loss, followed by high-resolution 3D light-sheet imaging for digital counting. Operating in the single-molecule regime enables precise quantification and straightforward multiplexing, as each molecule amplifies independently without inter-target competition. Using ten spectrally distinct fluorophores, this system detects ten independent targets simultaneously on a four-color imaging platform. By applying combinatorial fluorophore labeling, multiplexing is extended beyond twenty targets within a single reaction.

We demonstrate applications including a >20-plex respiratory pathogen detection panel and a 16-plex gene expression panel, both developed with minimal optimization. Quantitative results show concordance with single-plex assays and maintain high target specificity. Data from these panels were further analyzed using multivariate and high-dimensional visualization methods such as UMAP, analogous to approaches used in single-cell transcriptomic analysis, enabling intuitive interpretation of multiplexed results.

This single-molecule PCR framework establishes a scalable strategy for high-plex quantitative analysis with NGS-comparable precision. It provides a generalizable approach for accelerating biomarker validation and supports the development of multi-gene assays for cancer subtyping, prognostic scoring, pathogen detection, and other applications requiring rapid, high-plex quantification.

## Background

Multiplexing is inherently challenging in conventional qPCR and dPCR. Amplification bias, caused differences in amplification efficiency across targets, and spectral overlap between fluorescent dyes, both require extensive optimization to overcome. As a result, the number of distinct targets that are assayed in a single reaction is typically limited to fewer than ten.

For biomarker validation, such as post-RNA-seq studies, it is highly desirable to have a quantitation tool with the speed and cost-efficiency of qPCR/dPCR but with the prevision of next-generation sequencing (NGS).

## Results

### Overcoming challenges of high-order multiplexing with Countable PCR, a single-molecule quantification technique.

- In Countable PCR, DNA molecules are isolated across 30 million compartments in a clear gel-like matrix.
- Individual DNA molecules in the absence of competing target molecules, allowing for **bias-free PCR multiplexing** in a single reaction.
- The static nature of the matrix allows for serial imaging of a single compartment across many imaging channels to profile the unique optical signature of each single target molecule. **This enables the use of expanded fluorescent dye sets, even when spectral overlap exists.**

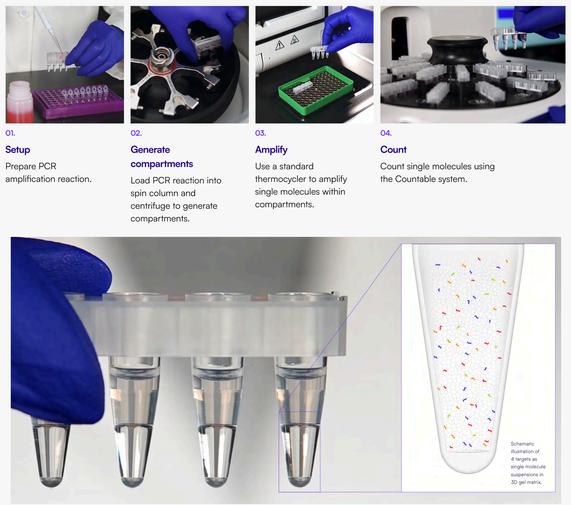



**Figure 1. Countable PCR workflow.** In Countable PCR, target molecules are distributed across 30 million compartments in an optically clear matrix. Every DNA molecule is physically separated into its own compartment and amplified without interference. This enables true single molecule counting, clean multiplexing, and data that remains precise across every run.

### Single-molecule counting with 10 different fluorophores on a 4-color imaging system.

All 30 million compartments are scanned in parallel across each imaging channel. Ten detection channels are configured to capture fluorescence signals from the dyes, which are sequentially imaged. The combined data from all channels generates and optical signature for every component.

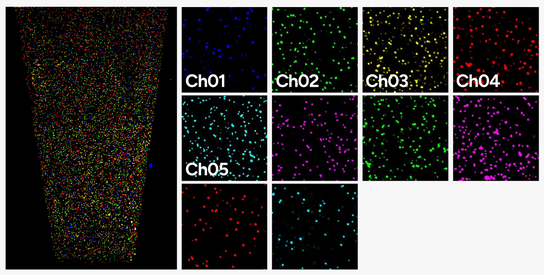



**Figure 2. Light sheet images of a Countable PCR reaction tube.** A composite image (left) shows the positive compartments from a mid-place slide of the tube. Zoomed-in images (right) display pseudo-colored positive compartments from each channel.

The optical signature from each compartment is unambiguously assigned to a specific dye. Even dyes with similar excitation wavelengths are able to be differentiated by imaging them in two distinct channels with different emission bandpasses, allowing accurate resolution of closely related fluorophores.

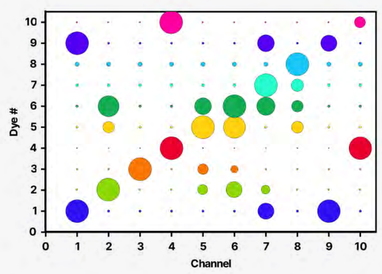



**Figure 3. Optical signature of a 10-dye reaction.** The signal intensity of each dye imaged at a unique excitation and emission channel is potted, where the size of each circle is proportional to the signal intensity normalized across each channel.

## Results, cont.

### Combinatorial labeling of target molecules expands multiplexing capacity.

To further expand multiplexing capability, it is possible to label amplicons derived from a single target, using multiple dyes to generate a unique optical signature, **theoretically extending the multiplex capability up to 45 targets** with dual labels involving combinations of 10 dyes.

**Table 1.** Possible number of multiplex combinations achievable by combinatorial labeling using k fluorescent labels per target.

| # of dyes (n) | $k = 1$ | $k = 2$ |
|---|---|---|
| **4** | 4 | 6 |
| **5** | 5 | 10 |
| **6** | 6 | 15 |
| **7** | 7 | 21 |
| **8** | 8 | 28 |
| **9** | 9 | 36 |
| **10** | 10 | 45 |

### High Plex Countable PCR as a Biomarker Validation Tool: Example of a 16-plex tissue signature gene expression panel.

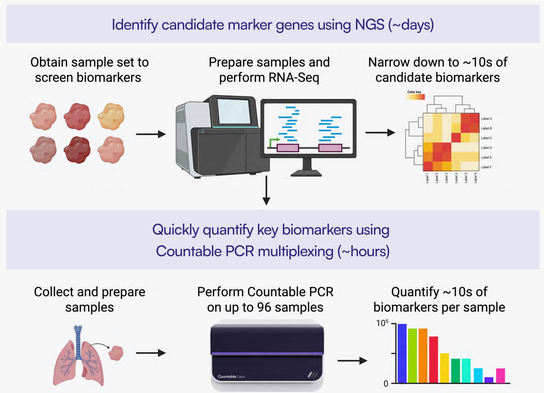
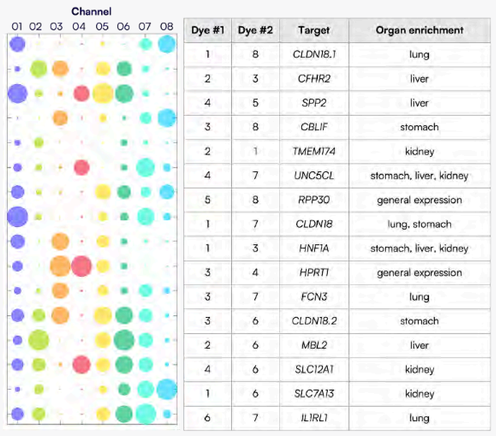



**Figure 4. Validate biomarkers using Countable PCR multiplexing.** Countable PCR can accelerate biomarker validation with high-plex quantitative analysis with NGS-comparable precision.




| Dye #1 | Dye #2 | Target | Organ enrichment |
|---|---|---|---|
| 1 | 8 | *CLDN18.1* | lung |
| 2 | 3 | *CFHR2* | liver |
| 4 | 5 | *SPP2* | liver |
| 3 | 8 | *CBLIF* | stomach |
| 2 | 1 | *TMEM174* | kidney |
| 4 | 7 | *UNC5CL* | stomach, liver, kidney |
| 5 | 8 | *RPP30* | general expression |
| 1 | 7 | *CLDN18* | lung, stomach |
| 1 | 3 | *HNF1A* | stomach, liver, kidney |
| 3 | 4 | *HPRT1* | general expression |
| 3 | 7 | *FCN3* | lung |
| 3 | 6 | *CLDN18.2* | stomach |
| 2 | 6 | *MBL2* | liver |
| 4 | 6 | *SLC12A1* | kidney |
| 1 | 6 | *SLC7A13* | kidney |
| 6 | 7 | *IL1RL1* | lung |

**Figure 5. Optical signature with combinatorial labeling.** The characteristic value of each dye combination at each channel is plotted, where the size of each circle corresponds to the optical intensity at each channel (left). Each target, with its organ specificity, is listed with the dyes used in their assays (right).

Counts were derived from whole tissue cDNA by mapping positive compartments onto the reference UMAP below.

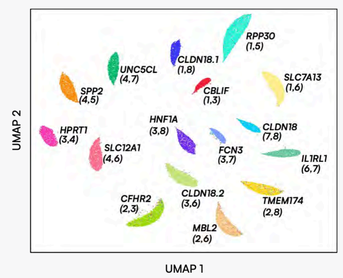



**Figure 6. Successful detection of 16 targets in a single Countable PCR reaction.** UMAP visualization of 16 separate Countable PCR reactions, each containing a synthetic DNA template of one of the target genes. Numbers next to the gene name indicate the dye numbers used in the combination.

## Results, cont.

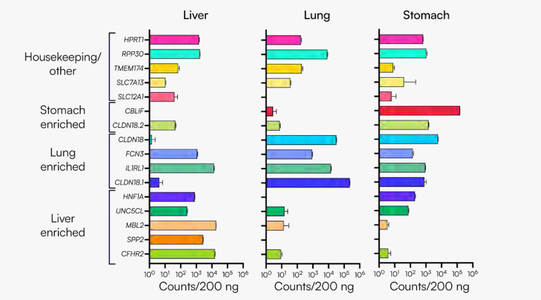



**Figure 7. Gene expression profiling of liver, lung, and stomach tissue using a 1-tube 16-plex panel.** Genes are grouped by the tissue in which they are most enriched or as housekeeping genes. The measured counts per 200 ng tissue (horizontal axis) are plotted for each gene (vertical axis).

### Quantification of common respiratory pathogens with a 22-plex Countable PCR panel.

Similarly, 8 dyes were used to develop a 22-plex of common respiratory pathogens and associated antibiotic resistance genes.

**Table 2.** Respiratory pathogens and common antibiotic resistance genes in the 22-plex panel.

| Cluster ID | Target |
|---|---|
| 1 | Metapneumovirus |
| 2 | Parainfluenza virus 1 |
| 3 | Legionella pneumophila |
| 4 | Parainfluenza virus 2 |
| 5 | Antimicrobial resistance gene: KPC |
| 6 | Parainfluenza virus 4 |
| 7 | Chlamydia pneumoniae |
| 8 | Klebsiella pneumoniae |
| 9 | Antimicrobial resistance gene: IMP |
| 10 | Proteus mirabili |
| 11 | Klebsiella oxytoca |

| Cluster ID | Target |
|---|---|
| 12 | Rhinovirus |
| 13 | Respiratory syncytial virus |
| 14 | Adenovirus |
| 15 | Escherichia coli |
| 16 | Serratia marcescens |
| 17 | Enterobacteria |
| 18 | Influenza_A |
| 19 | Staphylococcus aureus |
| 20 | Coronavirus 229E |
| 21 | Antimicrobial resistance gene: NDM |
| 22 | Bordetella parapertussis |

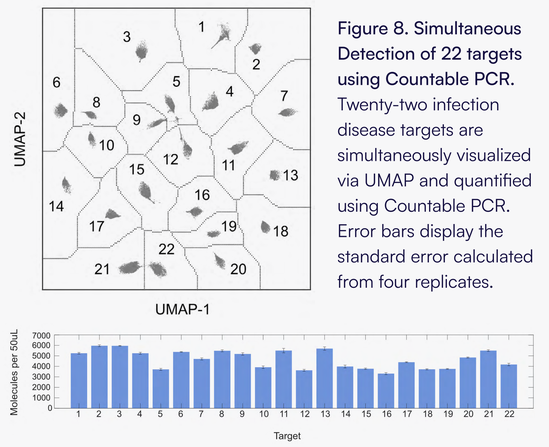



**Figure 8. Simultaneous Detection of 22 targets using Countable PCR.** Twenty-two infection disease targets are simultaneously visualized via UMAP and quantified using Countable PCR. Error bars display the standard error calculated from four replicates.

## Conclusion

Together, these findings establish Countable PCR as a platform capable of multiplexing beyond the limits of conventional PCR methods.

Countable PCR achieves high-order multiplexing through single-molecule compartmenting, which prevents amplification bias and ensures consistent quantitation across multiple targets.

Combinatorial labeling was applied to whole tissue cDNA using a 16-plex gene expression panel. The five-log dynamic range across all targets and isoform-level precision highlight both the multiplexing capacity and specificity of Countable PCR.

Similarly, combinatorial labeling was used to develop a 22-plex infectious disease panel, able to detect all targets simultaneously at high precision.

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.

## References

Lai, Janice H., et al. "New realm of precision multiplexing enabled by massively-parallel single molecule UltraPCR." bioRxiv (2023): 2023-10. doi: https://doi.org/10.1101/2023.10.09.561546

Learn more about how Countable PCR transforms multiplexing.

A Single-Molecule PCR Framework Bridging Low-Plex PCR and NGS for Biomarker Validation at 16-Plex and Beyond