Countable Labs.

Development of high-order, precision multiplexed PCR panels for gene expression biomarker validation using single-molecule counting.

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Abstract

Quantitative (qPCR) or digital PCR (dPCR) is commonly used to validate biomarkers following RNA-seq studies, often involving multiple gene markers. PCR measurement of panels of gene expression markers also forms the basis for various clinical diagnostic and prognostic tests. Multiplexing, combining several biomarkers in a single PCR reaction, improves efficiency and accuracy while reducing time, reagent use, and sample consumption. However, designing high-order multiplex PCR panels typically requires extensive optimization and iterative assay development. Having faster, more straightforward approaches to develop multiplexed PCR panels is highly desirable.

A major technical challenge of high-order multiplexing is balancing amplification efficiency across targets and resolving spectral overlaps of fluorophores used to detect each target. To overcome these challenges and open up new, more straightforward avenues for high-order multiplexing, we utilized Countable PCR, a single-molecule DNA quantification technique. Using the 4-color Countable system, we successfully resolved 8 distinct fluorescent dyes in one PCR reaction. By applying combinatorial labeling with 8 fluorophores, we developed a 16-target gene expression panel with marker genes specific to various human tissues. With minimal assay iteration and optimization, we demonstrated that the quantitative performance of the 16-plex panel matches that of individual assays with tissue specific cDNA, with gene expression levels varying over 6-logs.

This high-precision, high-order multiplexing method significantly expands the capabilities of PCR, dramatically reducing panel development time and accelerating validation studies following bulk- or single-cell RNA-seq. Further, it has the potential to eliminate or reduce the need for NGS in many biomarker quantification applications. Beyond validation studies, this approach holds strong potential for clinical assays that are based on panels of gene expression markers.

Background

Multiplexing is inherently challenging in conventional qPCR and dPCR. Amplification bias, caused by 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 10.

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 precision 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.**

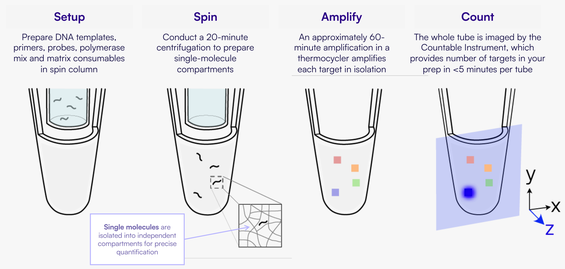


Figure 1. Schematic diagram of standard Countable PCR workflow. In Countable PCR, target molecules are distributed across 30 million compartments in an optically clear matrix. Post-PCR, positive compartments emit a fluorescent signal and are captured and quantified using 3D light sheet imaging.

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. Nine detection channels are configured to capture fluorescence signals from the dyes, which are sequentially imaged. The combined data from all channels generates an optical signature of intensity values for every compartment.

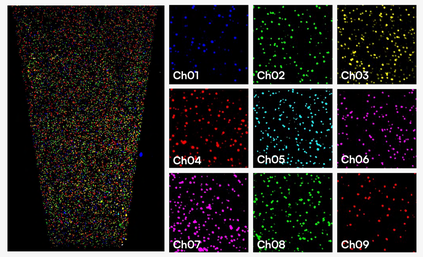


Figure 2. Light sheet images of a Countable PCR reaction tube. A composite image (left) shows the positive compartments from a mid-plane slice of the tube. Zoomed-in images (right) display pseudo-colored positive compartments from each channel.

The optical signature from each compartment are 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.

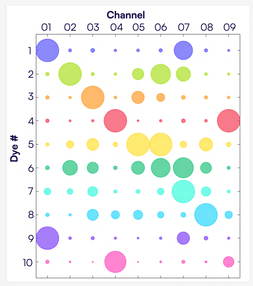


Figure 3. Optical signature of a 10-dye reaction. The signal intensity of each dye imaged at a unique excitation and emission channel is plotted, where the size of each circle is proportional to the intensity at each channel.

Results, cont.

Accurate quantification of 10 targets in a single Countable PCR reaction.

The optical signatures from the 9 channels generate a vector of intensity values for each positive compartment for a 10-plex reaction, each target labeled by one of the 10 unique dyes. These vectors were visualized using Uniform Manifold Approximation and Projection (UMAP), enabling the classification and counting of compartments corresponding to each target (**Figure 4A**). Counts were consistent whether a single target or all ten targets were present in the reaction (**Figure 4B**).

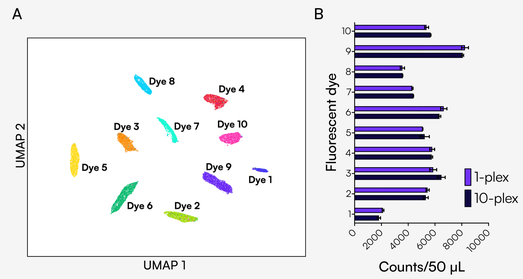


Figure 4. Quantification of targets in a 10-plex reaction. (A) UMAP visualization of optical signatures, showing separation of dye clusters. (B) Molecule counts (horizontal axis) from manual gating of UMAP clusters (vertical axis). N = 2. Counts obtained using a single 10-plex reaction is highly concordant with individual 1-plex reactions.

Combinatorial labeling of target molecules further expands multiplexing capacity.

To further expand multiplexing capability, it is possible to label a single target with multiple dyes to generate a unique optical signature, **theoretically extending the multiplex capability up to 45 targets.**

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

Measuring the gene expression signature of multiple organs with a 16-plex Countable PCR panel.

A 16 plex gene expression assay was developed by combinatorially combining 8 dyes.

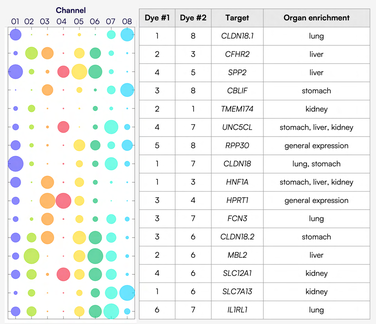
- **Organ identification.** This panel captures gene markers with expression patterns specific to lung, stomach, liver, and kidney for robust identification and quantification.
- **Isoform-level precision.** Identification of two isoforms of *CLDN18* unique to lung vs stomach.
- **Clinical/translational significance.**

Biomarkers: Expression patterns used to identify tissue origin such as for cancer.

Therapeutics: Relevance in immune signaling (*IL1RL1*), metabolism (*HNF1A*), and infectious disease susceptibility (*MBL2*).

Diagnostics: Relevance in diabetes (*HNF1A*), immunodeficiency (*FCN3*), and complement system disorders (*MBL2*).

Drug Targets: *SLC12A1* is a drug target involved with loop diuretics.



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 signal intensity of each dye combination at each channel is plotted, where the size of each circle is proportional to the intensity at each channel (left). Each target, with its organ specificity, is listed with the dyes used in their assays (right).

Results, cont.

Synthetic DNA templates were first used to generate a UMAP template of 16 targets.

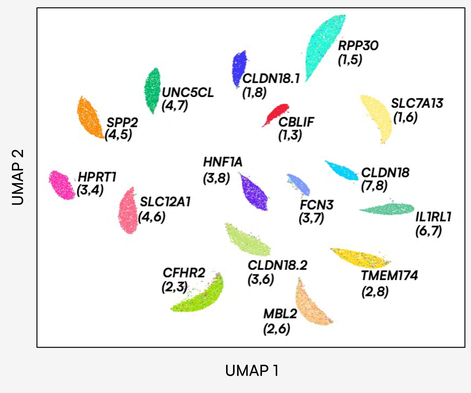


Figure 6. Successful detection of 16 targets in a single Countable PCR reaction. UMAP visualization of 16 separate Countable PCR reactions, each containing a synthetic DNA template of one of the target genes. Numbers next to the gene name indicate the dye numbers used in the combination.

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

- Distinct organ-specific expression signatures are measured in liver, lung, and stomach.
- Gene counts vary over 5-logs within a single tissue sample, quantifying a wide variety of genes, including transcription factors (*HNF1A*), housekeeping genes (*RPP30, HPRT1*), immune regulatory (*CFHR2, FCN3, MBL2, IL1RL1*), in a single reaction.
- Combinatorial labeling is specific, being able to resolve two different *CLDN18* isoforms between lung (*CLDN18.1*) and stomach (*CLDN18.2*)

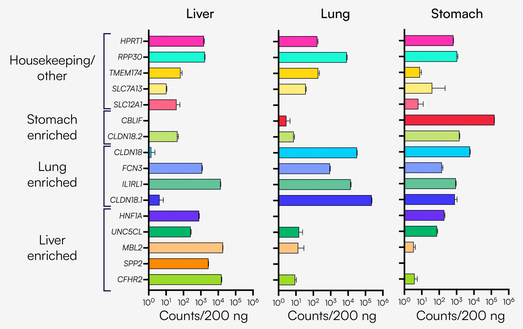


Figure 7. Gene expression profiling of liver, lung, and stomach tissue using 16-plex combinatorial labeling. Genes are grouped by the tissue in which they are most enriched or as housekeeping genes. The measured counts per 200 ng of tissue (horizontal axis) are plotted for each gene (vertical axis).

Conclusions

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.

Countable PCR enables incorporation of 10 optically distinct dyes on a four-color instrument.

Combinatorial labeling was applied to identify lung, stomach and liver tissue using a 16-plex gene expression panel. This panel demonstrated a five-log dynamic range across all targets and achieved isoform-level precision, highlighting both the multiplexing capacity and specificity of Countable PCR.

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.

Learn more about how Countable PCR transforms multiplexing.