Universal Multiplexing is a versatile, cost-effective solution for probing multiple targets with Countable PCR

Comparison of Universal Multiplexing vs. traditional TaqMan hydrolysis probes in Countable PCR demonstrates cost-effective multiplexing with similar performance

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Introduction

Multiplex PCR is a powerful technique that enables the simultaneous detection of multiple targets in a single reaction [1]. To date, the development of multiplex PCR assays has often presented challenges that have limited their widespread adoption. These challenges revolve largely around:

- Optimization of primer/probe pairs to avoid amplification bias and artifacts
- Prohibitive cost of scaling experiments with custom hydrolysis probes (HP) such as TaqMan (TM) probes

Countable PCR isolates each DNA molecule in its compartment within a gel-like matrix for independent amplification. Multiplexing is achievable with minimal optimization as there is no competition between targets within compartments. This alleviates the need for many rounds of optimization.reactions [4]. The result is very low VAF% sensitivity with good statistical confidence in a single reaction.

To overcome challenges related to cost, Countable Labs developed Universal Multiplexing (UM), a novel chemistry that eliminates the need for target-specific HPs. UM uses generic prefixed probe sequences with target-specific primers. Compared to HP-based approaches, UM significantly reduces assay design and run costs by eliminating expensive target-specific probes that require lengthy synthesis times.

In this study, we demonstrate the development of a 4-plex UM assay based on an assay originally developed using HP, and compare the performance of both on the Countable PCR platform.

Materials & Methods

Development of a Universal Multiplexing assay to demonstrate transferability from HP-based PCR assays

We used the Universal Multiplex Set A Kit (#KT0005) to create multiplex assays for up to 4 targets per reaction. **Table 1** lists the UM adapter sequences for the UM-1 to UM-4 probes within the kit.

Table 1. 4 UM adapter sequences in the Universal Multiplex kit.

Probe ID	Channel	Probe sequence
UM-1 Probe	Ch01	5'-TAGAAGGCACAGTCGAGG-3'
UM-2 Probe	Ch02	5'-CAGAAGACGGCATACGAGAT-3'
UM-3 Probe	Ch03	5'-ACCGTAGAGTCCGAGCAA-3'
UM-4 Probe	Ch04	5'-GAAGCGTTTATGCGGAAGAG-3'

Table 2. Primer sequences used for the 4-plex UM assay. UM adapters (underlined) were appended to the 5' end of one of the gene-specific primers to generate a UM primer. No modification is required for Non-UM primer.

Target	Probe/primer	Probe ID and sequence for primers
RPP30	UM probe	UM-1 Probe
	UM primer	5'-TAGAAGGCACAGTCGAGG AGATTTGGACCTGCGAGCG-3'
	Non-UM primer (R)	5'-GAGCGGCTGTCTCCACAAGT-3'
JAK2	UM probe	UM-2 Probe
	UM primer	5'-CAGAAGACGGCATACGAGATAAG CTTTCTCACAAGCATTTGG-3'
	Non-UM primer (R)	5'-AGGCATTAGAAAGCCTGTAGTTTTA CTT-3'
RAD51	UM probe	UM-3 Probe
	UM primer	5'-ACCGTAGAGTCCGAGCAA TTGGTGACTTTTGCCCATATTA-3'
	Non-UM primer (R)	5'-GGTTGTGGTCAACAAAATACGT-3'
MET	UM probe	UM-4 Probe
	UM primer	5'-GAAGCGTTTATGCGGAAGAG CAATGTGAGATGTCTCCAGCAT-3'
	Non-UM primer (R)	5'-GGGAACTGATGTGACTTACCCTA-3'

Table 2 shows the sequences of both UM primers and Non-UM primers designed for each of the 4 targets in one of our 4-plex assays, demonstrating how these primers were derived from forward and reverse primers from a previously designed HP-based assay.

Following best practices for multiplex PCR assay design, we used oligo analysis tools to conduct in silico analysis to check for self-interactions (such as hairpin formation) within oligos — including those with appended UM adapters — and cross-interactions between all primers, probes, and amplicons to prevent non-specific signal generation. We particularly focused on evaluating potential primer-dimer formation to minimize false signal generation.

Countable PCR reactions were set up and analyzed according to the Countable PCR Reaction Preparation User Guide (IFU004) and Countable System Instructions for Use (IFU003) as shown in **Table 3**.

Table 3. Countable PCR reaction set up. To generate optimal signal, excess non-UM primer is included as compared to UM primer, following the guidelines of preparing 50x UM Primer Mix outlined in the Countable PCR Reaction Preparation User Guide.

Reagent	Catalog No.	Amount per 50µL	Final Concentration
Nuclease-free water	N/A	28.5 µL	N/A
4X Countable PCR Mix	KT0004 (PR0004)	12.5 µL	1X
50X UM 1—4 Probes	KT0005 (PR0006- PR0009)	1 µL/Probe, 4 µL total	1X
50X UM Primer Mix (per target)	N/A	1 µL/target, 4 µL total	1X (UM Primer-80 nM; Non-UM Primer-400 nM)
Human genomic DNA	N/A	1 µL	Variable

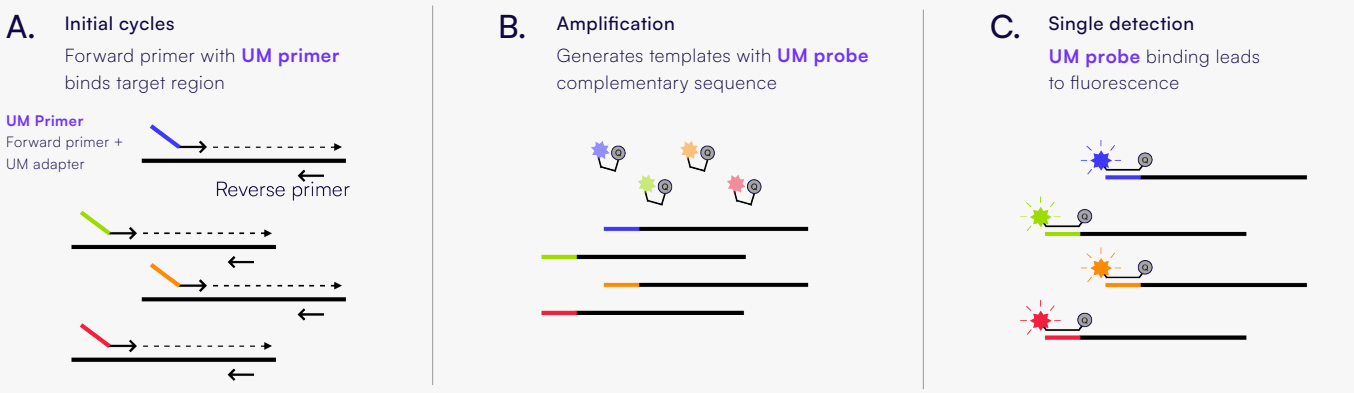


Figure 1. Principle of Universal Multiplexing chemistry. (A) During initial cycles of PCR, the UM primer (a forward primer with UM adapter (in blue)) binds to the template and extends. (B) In subsequent PCR cycles, the non-UM primer (a reverse primer, unmodified) binds to the sense forward template (now with UM adapter) and extends to create an UM probe complementary sequence. (C) The detection of the target amplicon occurs via hybridization of the UM probe to the target amplicon.

Results

Principle of Universal Multiplexing chemistry

Figure 1 illustrates the assay principle of Universal Multiplexing chemistry. In UM, one primer is appended with a UM adapter sequence. During initial PCR cycles, the primer with the UM adapter binds to the template and extends [1A]. In subsequent cycles, the non-UM primer (typically R primer) binds to the forward template (now with UM adapter) and extends to create a UM probe complementary sequence [1B]. Detection occurs when the UM probe hybridizes to the probe binding site within the amplicon [1C].

UM chemistry delivers the same counting performance as hydrolysis probe assays at a fraction of the cost

To demonstrate that UM is a robust substitute for HP-based assays, we compared the Countable PCR performance of a UM 4-plex assay to that of a 4-plex HP-based assay targeting the same genes. Counts from UM assay matched those from HP-based assay closely, with less variation in total counts, as shown in Figure 2.

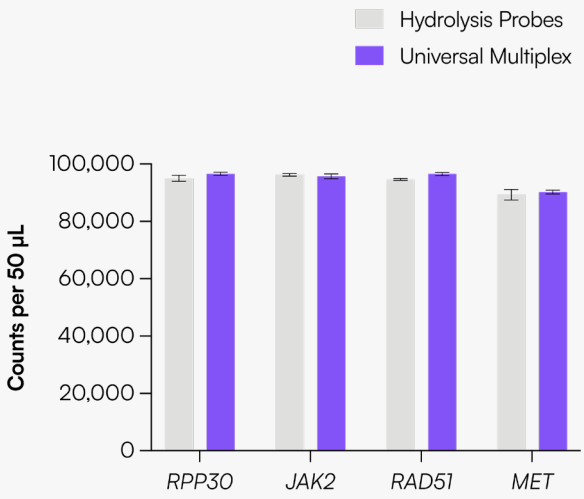


Figure 2. Comparison between counts per target obtained from a 4-plex UM assay versus a 4-plex HP assay targeting the same genes. Counts averaged across 4 replicates per condition are shown. Standard deviations were used for the error bars. The difference between HP and UM in each assay was less than 2% in all targets.

Table 4 shows how the use of Universal Multiplexing reduces costs compared to using hydrolysis probes. From design to the first experiment, the 4-plex UM assay used in this study took less than 1 week. Since only standard desalted primers were required, the oligo synthesis cost was approximately $120 for the UM 4-plex assay—about ten times less than a 4-plex HP-based assay ($1200-2000). The oligo synthesis took only 2-3 days, instead of 2-4 weeks for custom hydrolysis probes.

Table 4. Comparison of HP and UM chemistries.

	Hydrolysis probes	Universal Multiplex
Specificity	High	High
Mutiplexing	Yes	Yes
Cost of oligos + probes	**High** Forward primer: ~$10 Reverse primer: ~$10 Probe: ~$250 - $500* Total: ~$300-$500 per target	**Low** UM primer: ~$20 Non-UM primer: ~$10 Total: ~$30 per target
Turn-around time from oligo synthesis	2-4 **weeks**	2-3 **days**
Pre-optimized signal generation	No	Yes

* Depending on choice fo flourophore

Because the fluorescent signals in the UM chemistry come from a pre-optimized UM probe mix, the signal and noise characteristics of the assay are independent of primer sequences. In contrast, with HP-based assays, signal-to-noise characteristics depend on probe sequence design (6) and at times vary between different batches of synthesized probes.

UM achieves robust counting performance from 1-plex to 4-plex, across 6-logs

We compared 1-plex, 2-plex, 3-plex, and 4-plex configurations in Countable PCR using 4 UM assays targeting different gene sequences.

Using human gDNA as a template, we demonstrated that counts remained consistent regardless of plex number (Figure 3). There was no amplification bias in the presence of other targets for Countable PCR—a phenomenon commonly observed in qPCR or even in digital PCR when one partition contains multiple templates. The assay noise was also low (0 for NTC samples), as evidenced by the non-detection of counts for NTC samples within the 4-plex condition.

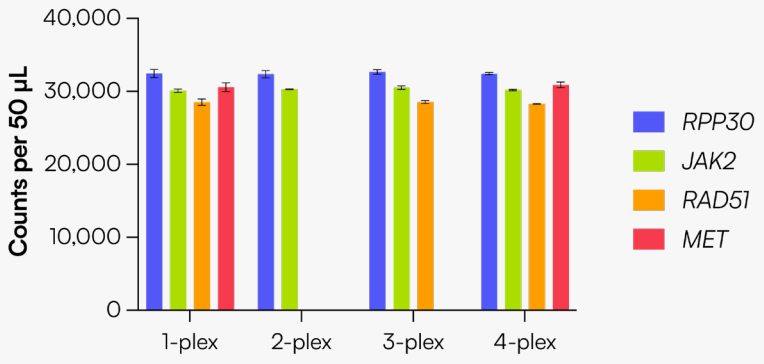


Figure 3. Comparison of 1-plex, 2-plex, 3-plex, and 4-plex in Countable PCR targeting 4 different human genes. The counts for all four targets remained consistent, regardless of whether other targets were present or not. Counts averaged across 3 replicates per condition are shown. The error bars represent standard deviations. The difference between 1-plex and 4-plex was -0.13%, 0.35%, -0.84% and 1.03% for RPP30, JAK2, RAD51 and MET, respectively.

We further tested the same 4-plex UM assay with DNA templates diluted across a 6-log range of serial dilutions, with roughly equal amounts of templates per target. As shown in Figure 4, the assay demonstrated robust, reproducible quantification of four different targets from across a 6-log dynamic range.

This capability allows for the detection of both abundant and rare targets in the same sample. In applications such as in gene expression analysis, these comparisons are often conducted in separate qPCR assays with DNA intercalating dyes (single-plex).

UM offers flexibility in multiplex assay design

The UM adapter sequences were carefully designed not to interact with genome sequences of any common species, while preserving the same counting performance across adapter designs. Any UM adapter sequence will convert a primer into a UM primer when added, as long as attention is paid to avoiding hairpin formation resulting from the adapter addition.

To demonstrate this flexibility of UM adapter choices, we evaluated 3 targets with different expression levels: GAPDH (high expression), CD3E (medium expression), and CD1A (low expression) using human cDNA as a template. UM-1, UM-2, and UM-4 probes were attached to forward primers for each target and permuted as shown in Figure 5. Counts remained constant for all targets regardless of the UM probe chosen, and despite varying expression levels, the coefficient of variation remained low across all samples. This assay demonstrated the flexibility of UM for multiplex target design; when designing multiplex assays for targets that have multi-log range variability in expression levels, there are no restrictions on which channel may be used for different target genes.

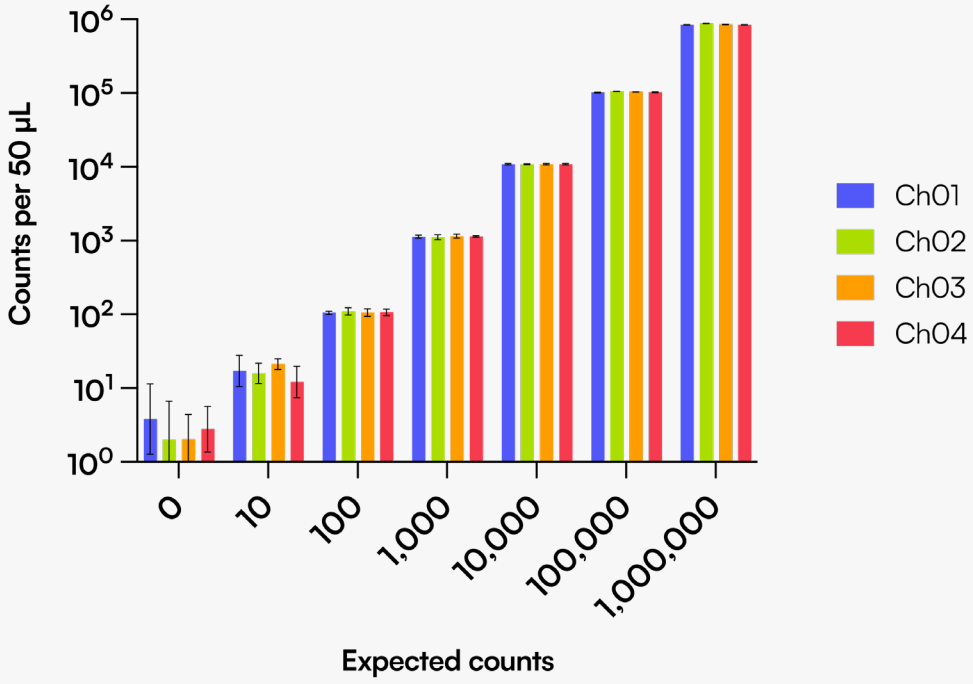
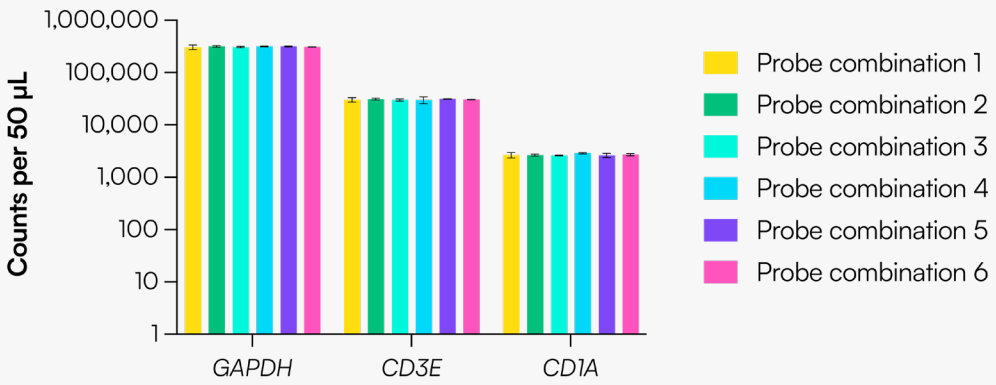


Figure 4. Dynamic range of UM with 4-plex assay. 4 different targets were quantified using UM probes across dilution series from 0 to 1,000,000 of template (N=8). The error bars represent standard deviations. Even in multiplexed conditions, UM showed a linear increase in counts per target across a 6-log range.



Probe Combination	1	2	3	4	5	6	Avg. Counts	%CV
GAPDH	UM-1	UM-4	UM-4	UM-1	UM-2	UM-2	312,711	1.46
CD3E	UM-4	UM-1	UM-2	UM-2	UM-4	UM-1	30,626	1.94
CD1A	UM-2	UM-2	UM-1	UM-4	UM-1	UM-4	2,686	3.91

Figure 5. Probe permutation in high/medium/low expression targets. Three targets with varying expression levels were quantified using different combinations of UM probes as shown in the table above. The error bars represent standard deviations (N=4). The assay performance remained consistent regardless of the UM adapter choice or expression level. %CV was calculated across samples with the same targets, regardless of probe combinations.

It is also possible to use UM probes in combination with HP within the same experiment. This flexibility of assay design strategy is useful in applications where certain gene targets benefit from the additional specificity conferred by hydrolysis probes. As long as there is no significant interaction between oligos in the multiplex assay, HP and UM function effectively together in a single tube. To demonstrate, we constructed a 4-plex assay in which two of the targets were assayed with UM chemistry, and the remaining two were assayed with HP, with non-overlapping channel assignment (Figure 6). The counts measured for each of the targets are consistent across different assay design configurations (all UM, or a combination of HP and UM).

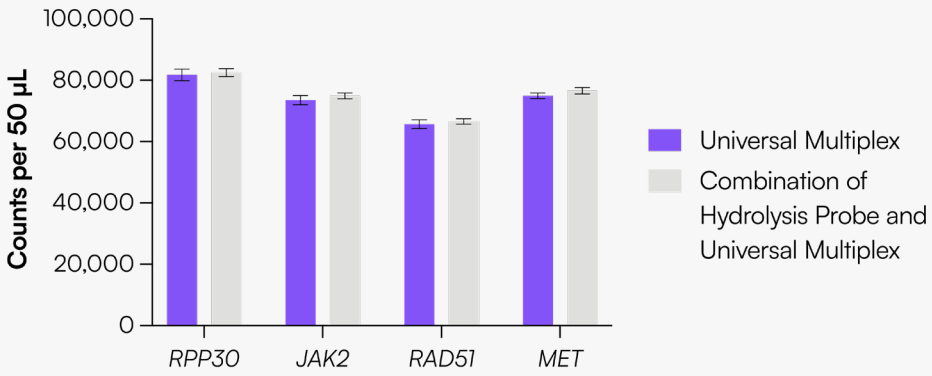


Figure 6. Combination of a 4-plex assay utilizing HP chemistry for two targets and utilizing UM. The error bars represent standard deviations (N=4). Each target can be quantified without interference, either by all UM or a combination of UM and HP. For the combination with HP, RPP30 and JAK2 were detected by HP, and RAD51 and MET were measured by UM.

Conclusion

We demonstrated that UM delivers the same multiplexing and counting performance as HP-based assays in Countable PCR, but at a fraction of the cost. When integrated with Countable PCR, UM provides consistent counting precision across a 6-log range and for up to 4-plex experimental design, irrespective of probe sequence or expression level. In this performance benchmarking technical note, we show:

- Universal Multiplexing (UM) enables multiplex assays for up to 4 channels at approximately 1/10 the cost of conventional hydrolysis probe multiplexed assays
- By using only standard unmodified primers, UM accelerates development timelines of multiplexed PCR reactions to days, from initial design to the first working experiment
- UM delivers the same performance and specificity as multiplexed HP assays
- UM probes amplify cleanly across 6-log target concentration, without bias based on probe color/channel
- Countable PCR extends bias-free amplification multiplexing to HP-based assays as well

Further, Universal Multiplexing represents a dramatic decrease in both the monetary cost and time spent optimizing multiplex experiments for those already using hydrolysis probes. A four-plex experiment with UM costs ~$120 and takes 2-3 days to develop, compared to $1500-2000 and 2-3 weeks for HPs.

UM is ideal for applications requiring quick turnaround times and rapid assay design iteration, or for studies with limited sample numbers that don't warrant the expense of HP-based assays. Combined with the broad dynamic range of the Countable System, where each target occupies a separate compartment, eliminating amplification bias between different targets, UM provides a straightforward and cost-effective multiplexing solution.

References

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