Countable Labs.

Countable PCR™ Reaction Preparation User Guide

For use with the Countable System

For Research Use Only. Not for Use in Diagnostic Procedures.

Legal Notices

IFU004 Rev 1.0, June 2025

Countable Labs, Inc.
1810 Embarcadero Rd, Suite 200
Palo Alto, CA 94303, United States
www.countablelabs.com
success@countablelabs.com

Notices

Duplication and/or reproduction of all or any portion of this document without the express written consent of Countable Labs is strictly forbidden. Nothing contained herein shall constitute any warranty, express or implied, as to the performance of any products described herein. Any and all warranties applicable to any products are set forth in the applicable terms and conditions of sale accompanying the purchase of such product. Countable Labs provides no warranty and hereby disclaims any and all warranties as to the use of any third-party products or protocols described herein. The use of products described herein is subject to certain restrictions as set forth in the applicable terms and conditions of sale accompanying the purchase of such product. Countable Labs may refer to the products or services offered by other companies by their brand name or company name solely for clarity, and does not claim any rights in those third-party marks or names. The use of products described herein is subject to terms that have been agreed to in writing between Countable Labs and the user. All products and services described herein are intended FOR RESEARCH USE ONLY and NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Intended Use

All products and services described herein are intended for research use only and not for use in diagnostic procedures.

Revision History

Document Revision	Revision Date	Description
IFU004 Rev 1.0	June 2025	Initial release

Table of contents

CHAPTER 1

Welcome to Countable PCR™

Innovations

Countable PCR™ is a next-generation genomics system that delivers high-sensitivity, high-resolution nucleic acid quantification using direct single-molecule counting. Whether your application is rare variant detection, gene expression analysis, or next-generation sequencing (NGS) assay validation, Countable PCR provides direct, interpretable data with high precision.

Single molecule isolation—the foundation of Countable PCR

Countable PCR is built on the principle of physically separating individual target molecules before amplification. This is achieved through the Countable Matrix—a gel-like structure formed inside the PCR tube using a simple, centrifugation-based spin column workflow.

During matrix formation, target molecules are randomly and evenly distributed in 3D space within the tube. Each molecule is isolated within its own domain and undergoes amplification independently, without competition or interference from other templates.

This physical isolation enables:

- Single-molecule resolution
- Uniform amplification efficiency
- Clean, compartmentalized signal for direct counting

Simplified because single molecules behave independently

Conventional PCR, quantitative PCR (qPCR), and digital PCR (dPCR), often requires extensive optimization to reduce signal interference and balance amplification efficiency across targets. Countable PCR removes these constraints by isolating target molecules before amplification. Each molecule behaves as if it were in its own reaction. As a result, your panel development can be significantly accelerated.

This means:

- **Existing single-plex primer designs** can often be reused with minimal modification if there are no significant primer-dimer issues.
- **Multiplexing becomes easier** as targets no longer compete for reagents or interfere with each other.
- **Balancing and normalization are reduced**; single-molecule amplification minimizes PCR bias.

High-precision quantification enabled by direct molecule counting

In qPCR, quantification is based on fluorescence intensity and standard curves. In dPCR, it relies on Poisson estimation from partitioned droplets and microwells. Countable PCR eliminates both approaches by imaging and directly counting molecules after amplification.

This delivers:

- **Absolute quantification** — no standards or curve fitting
- **High statistical precision** — tighter coefficient of variation (CV) with fewer replicates
- **Wide dynamic range** — detect both low- and high-copy targets in the same tube
- **Reproducibility** — consistent results across wells, runs, and users

Countable PCR gives you true counts—not estimates—making your data easier to trust and compare across experiments.

The Countable PCR solution

Spin column-based setup for high-efficiency, high-volume precision

Countable PCR is built on a non-microfluidic, spin column-based sample preparation workflow. Unlike droplet- or microwell-based dPCR systems, Countable PCR uses standard PCR tubes and centrifugation to create the reaction environment, enabling efficient, dead-volume-free sample processing at large volumes.

At the core of this setup is the **Matrix Column Strips**, a single-use column that fits directly into a customized PCR tube. When centrifuged, it forms a structured gel matrix (the Countable Matrix) within the tube, where the target molecules become physically isolated before amplification.

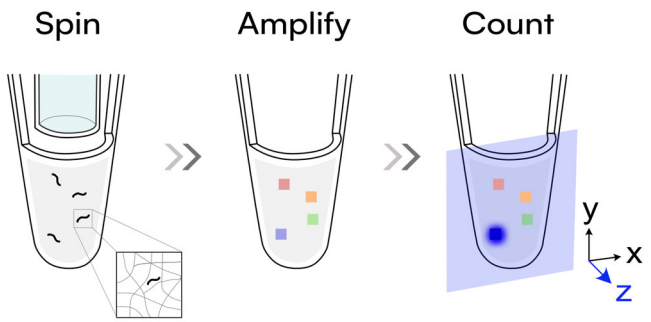
This enables a **wide dynamic range**—making Countable PCR ideal for workflows that require both high sensitivity and high capacity, such as rare-variant detection in cfDNA or dual-quantification of low- and high-abundance genes.

Key benefits:

- Process up to 35 µL of DNA input per reaction
- **Compatible with pre-amplified reactions** for enrichment of rare signals before counting
- **No dead volume** as the entire reaction is spun through and retained in the matrix
- **Visual dye indicator** confirms successful matrix formation before amplification
- Quantify up to 1 million molecules in a single reaction

One-tube workflow for closed, contamination-free precision quantification

Countable PCR is designed around a familiar PCR tube format—but with significantly more quantitative resolution built in. The entire process takes place inside a single sealed container, minimizing contamination risk and eliminating the need for sample transfers.



The Countable PCR workflow

1. **SPIN** — Place the Matrix Column Strip into the Matrix Tube Strip and centrifuge. The reaction mix is converted into a 3D gel matrix with physically isolated molecules.
2. **AMPLIFY** — Run thermal cycling directly in the same sealed PCR tube. No droplet generation, emulsion formation, or oil overlays are required.
3. **COUNT** — Image the sealed tube using the Countable System. 3D light sheet imaging maps fluorescent signals from amplified molecules throughout the entire matrix volume.

   Instructions for imaging setup, file handling, and data interpretation are provided in the *Countable System Instructions for Use* (IFU003).

Key benefits:

- **Closed-tube system**—no open transfers or fluid handling post-setup
- **No cross-contamination or sample loss**
- **No complex microfluidics, or cartridges**
- **Direct digital counting of each amplified molecule**—no Ct values, no Poisson estimation

Supported multiplex chemistries

Countable PCR supports two flexible, high-performance chemistries for multiplex detection—**hydrolysis probes (HP)** and **Universal Multiplexing (UM)**. Each chemistry offers distinct advantages, depending on your assay design goals, cost sensitivity, and turnaround requirements.

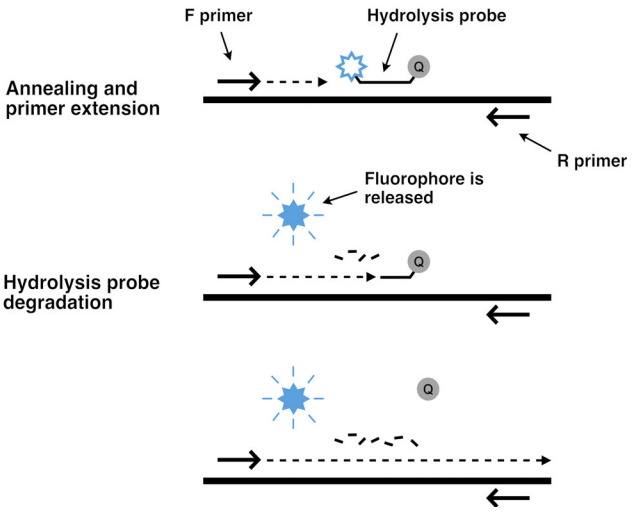
DNA intercalating dyes (e.g., SYBR Green) are not supported due to incompatibility with Countable PCR's single-molecule imaging and counting workflow. However, if you are currently using SYBR-based assays, transitioning to Universal Multiplexing is straightforward—it only requires a short adapter sequence added to one of your primers. The rest of your assay design can remain unchanged, making it easy to migrate existing workflows with minimal revalidation.

Key benefits of multiplexing on Countable PCR:

- **Lower assay cost** — fewer reactions, fewer consumables
- **Preserved sample** — ideal for your low-input or precious samples
- **Increased sensitivity** — detect rare events by expanding assay coverage without diluting input across reactions

Hydrolysis probes (HP)

Hydrolysis probes are widely used in qPCR and dPCR for their specificity and high sensitivity. They consist of a target-specific oligo with a **5' fluorophore and 3' quencher**, typically used alongside forward and reverse primers.

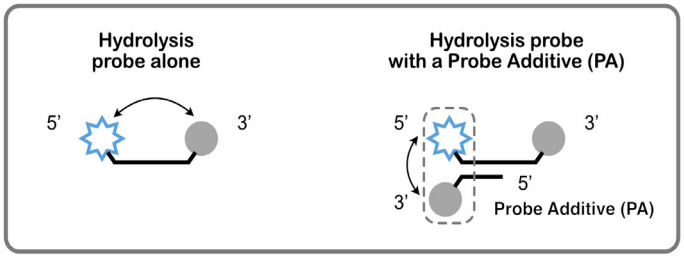


Detection principle

During amplification, the DNA polymerase's 5'→3' exonuclease activity cleaves the bound probe, releasing the fluorophore from the quencher. This separation produces a measurable fluorescence signal specific to the target amplicon.

Considerations for Countable PCR when using HP

In a single-molecule context, background fluorescence from incomplete probe quenching can reduce the ratio of signal-to-background fluorescence intensities and affect counting accuracy.



To mitigate this, we strongly recommend using a **probe additive (PA)**—an oligo with a 3' quencher and partial complementarity to the 5' end of the HP. This enhances quenching efficiency and suppresses background fluorescence.

For a list of compatible fluorophores to be used for the HP chemistry, see Appendix C: Fluorophore compatibility of hydrolysis probes on page 23. For help with an HP + PA assay design, see Appendix D: Probe additive design on page 23, or contact technical support at success@countablelabs.com.

Universal Multiplexing (UM)

Universal Multiplexing (UM) is a novel chemistry developed by Countable Labs, which enables efficient multiplex detection without requiring target-specific hydrolysis probes.

Advantages of UM:

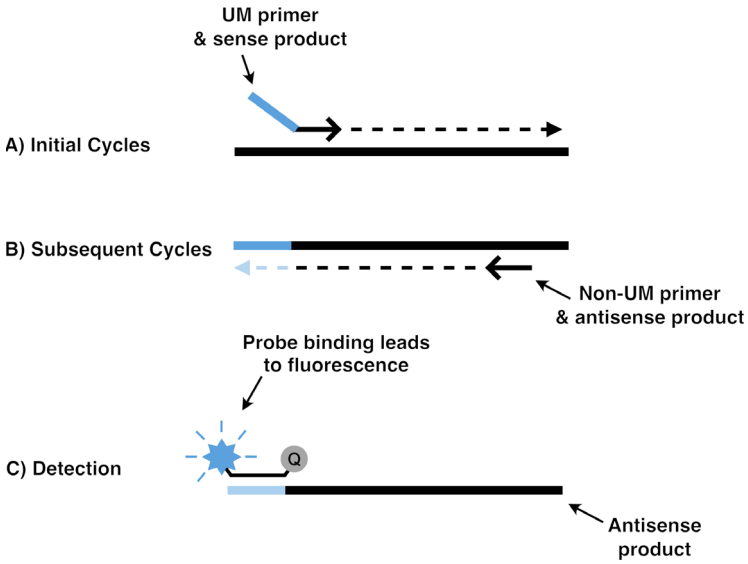
- No custom probe design required
- Lower assay development cost
- Faster assay turnaround
- Supports up to **four targets per reaction**
- Compatible with the **optional Universal Multiplex Set A Kit** (Cat # KT0005), which includes UM Probes UM-1 through UM-4.

UM assay design overview

1. Design your forward and reverse PCR primers as you normally would against your target.
2. Add the UM adapter to the 5' end of one primer (designated the UM primer).
3. Leave the other primer unmodified (the Non-UM primer).
4. Order both primers as standard desalted oligos, or HPLC-purified for high purity.
5. Combine with the matching UM probe from the Universal Multiplex Set A Kit (Cat # KT0005).

Instead of designing a custom probe for each target, UM uses a set of universal probes (UM-1 to UM-4) of the Universal Multiplex Set A Kit (Cat # KT0005), each linked to a distinct fluorophore. These probes are used in combination with standard primers that include a short UM adapter sequence added to the 5' end of one of your primers per assay. See Appendix D: Probe additive design on page 23 for the sequences of the UM adapters.

How UM works



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

CHAPTER 2

Getting ready for your Countable PCR experiment

Countable PCR kit configurations

For safety information on reagents, refer to the Safety Data Sheets, which can be found at countablelabs.com.

Cat #	Kit	Description
KT0001	Countable Matrix Consumables Kit	Single-use consumables for Countable matrix formation. Supports 192 reactions. Kit includes: • Matrix Column Strips • Matrix Tube Strip Seals • Matrix Tube Strips and Caps Storage temperature, 18°C to 25°C
KT0002	Countable Swing Bucket Kit	Swing bucket inserts compatible with Eppendorf 5430/ 5430R centrifuge fitted with S-24-11-AT swing bucket rotor. Each spin holds 48 samples (8 samples x 6 buckets). Kit includes: • Countable Swing Buckets (x6) • Countable Swing Bucket Carrier • Countable Balance Strips (x4) Storage temperature, 18°C to 25°C
KT0003	Countable Matrix Kit	Reagents for Countable Matrix formation. Supports 192 reactions. Kit includes: • MR01 Matrix Reagent • MR02 Matrix Reagent • MR03 Matrix Reagent Storage temperature, 18°C to 25°C
KT0004	4X Countable PCR Mix Kit	PCR mix for 50-µL reactions. Compatible with hydrolysis probes and Universal Multiplex chemistries. Supports 192 reactions per channel per kit. Kit includes: • 4X Countable PCR Mix • 50 mM $MgCl_2$ Storage temperature, —25°C to —15°C

Cat #	Kit	Description
KT0005	Universal Multiplex Set A Kit	Contains UM probes 1—4 for 4-plex multiplexing chemistry using standard primers—no custom probes required. Designed for compatibility with Countable PCR Mix. Supports 192 reactions per kit. Kit includes: • 50X UM-1 Probe • 50X UM-2 Probe • 50X UM-3 Probe • 50X UM-4 Probe Storage temperature, —25°C to —15°C
KT0009	Countable Control Assay Kit	Positive control assay kit for Countable PCR. Required for new user training. Kit includes: • Countable Control Templates • Countable Control Primers Storage temperature, —25°C to —15°C

Required equipment

The items in the following tables have been tested by Countable Labs and perform optimally with the Countable System. Substituting materials may adversely affect the performance of the system.

Centrifuge

Manufacturer	Component	Cat #
Eppendorf	Centrifuge 5430/R	022620584 (5430 with keypad) 022620667 (5430R with keypad) 022620596 (5430 with rotary knobs) 022620689 (5430R with rotary knobs)
Eppendorf	Rotor S-24-11-AT	5427757007

Compatible thermal cyclers

Manufacturer	Component	Cat #
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module OR	1851197
Bio-Rad	PTC Tempo Deep well Thermal Cycler	12015392

Recommended consumables and reagents

Consumables and reagents

Manufacturer	Component	Cat #
Rainin	Pipettes: • P2 • P20 • P200 • P1000 • 8-channel P200	 • 17008648 (PR-2) • 17008650 (PR-20) • 17008652 (P-200) • 17008653 (P-1000) • 17013805 (L8-200XLS)
Rainin	Pipette tips: • Tips LTS 200 µL Filter RT-L200FLR • Tips LTS 1 mL Filter RT-L1000FLR • Tips LTS 20 µL Filter RT-L10FLR	 • 30389240 • 30389213 • 30389226
Eppendorf	DNA LoBind Tubes, 1.5 mL	022431021
Millipore	Ultrafree - MC - VV Centrifugal Filters Durapore PVDF 0.1 µm	UFC30VV00
Teknova	DNA Suspension Buffer	T0220

Best practices

This section outlines key recommendations to ensure the accuracy, consistency, and integrity of your Countable PCR results. We recommend following these tips and practices to minimize the risk of contamination, signal loss, or reaction failure.

Contamination control

Preventing cross-contamination

- Always use **fresh, disposable pipette tips** for every sample and reagent.
- Use **barrier or aerosol-resistant tips** to prevent airborne contamination.
- Set up **negative controls (NCs)** or **no-template controls (NTCs) before** adding any experimental or training samples.
- Maintain **physical separation** between clean and template-containing work areas.

Experimental controls

NC or NTC controls

Include negative controls (NCs) and no-template controls (NTCs) in every run to monitor background signal, contamination, and non-specific amplification. Multiple replicates are recommended to accurately define the false-positive threshold.

Training samples

Always include one training sample per channel per run to calibrate the system for accurate single-molecule classification, especially in low-abundance samples. Training samples should closely match your experimental DNA in size and concentration.

For detailed guidance on control setup, see Appendix A: Experimental controls on page 22.

DNA sample integrity and input

DNA sample handling

- Design experiments in **multiples of four**. Each Tube Strip holds four reactions; fill unused tubes with **nuclease-free water** to match sample layout.
- For **high DNA input volumes** (up to 35.5 µL), use **low-EDTA or EDTA-free buffers** (e.g., Teknova DNA Suspension Buffer, PN T0220) to prevent PCR inhibition.
- Avoid introducing **bead contamination** from magnetic bead-based DNA preps.
- Consider a **filtration step** using Millipore Ultra-free MC-VV filters (0.1 µm PVDF, PN UFC30VV00) at 12,000 x g for 2-5 minutes.

DNA sample type and compatibility

- Validated sample types include: **cfDNA**, **cell line DNA**, **FFPE DNA**, **viral DNA**, and **cDNA** from RNA.
- For **large DNA fragments** (e.g., genomic DNA), we recommend **fragmentation** (enzymatic or mechanical) before use to improve counting accuracy.
- Maximum tested DNA input is **1 µg** per 50-µL reaction; higher amounts may be possible but should be empirically tested for your assay.

Primer and probe recommendations

- Use **HPLC-purified primers and probes** for high-specificity applications to minimize synthesis-related artifacts.
- For assays using hydrolysis probes (HP), consider including a **probe additive (PA)** to suppress background and enhance signal-to-noise.
- For Universal Multiplex (UM), follow adapter design guidelines and match probes from the Universal Multiplex Kit for optimal signal separation.

Matrix handling and tube care

- Keep Matrix Tube Strips **upright** throughout the workflow to protect matrix stability.
- **Avoid shaking**, **vortexing**, **dropping**, or **abruptly handling** after matrix formation.
- Minimize **dust and fiber accumulation** on the Matrix Tube Strips. Wipe outer surfaces with **lint free wipes** before imaging.
- Cover or reseal any open consumables or peel-packs when not in use to avoid airborne contamination.

Pipetting accuracy and reagent handling

Viscous Reagents (e.g., 4X Countable PCR Mix)

- Pipette **slowly and steadily**—avoid bubbles during aspiration and dispense.
- When instructed, pipette against the inner wall of the Column Strip tube when making the amplification mix.
- **Do not pipette past the first stop**.
- **Visually inspect** the tip to confirm complete volume transfer.
- **Pre-wet tips** to improve pipetting consistency.
- Use low-retention tips and **low-retention DNA tubes** to prevent sample loss.
- For easier pipetting, allow viscous reagents to reach **room temperature** before use.

General reagent handling

- **Thaw and mix all reagents thoroughly** before use.
- **Calculate 10% excess** for each reagent to account for pipetting losses.
- Avoid pipetting **<2 µL volumes**. If needed, dilute samples to ensure accurate handling.

CHAPTER 3

Procedure

Prepare amplification mix

1. Start by getting everything ready at the bench. All reagents used in the amplification mix should be thawed at room temperature.

   Once thawed, gently vortex each tube for 2—5 seconds until the solution looks uniform. Briefly spin down the tubes to collect the liquid at the bottom.

2. Use a low-retention tube for DNA to prepare the mix. Follow the table below based on your assay chemistry.

For Universal Multiplexing

Reagents	Cat #	Per 50 µL reaction	Final conc.
Nuclease-free water	—	To 50 µL	—
4X Countable PCR Mix	KT0004 (PR0004)	12.5 µL	1X
50X UM 1—4 Probes[1]	KT0005 (PR0006-PR0009)	1 µL/probe	1X
50X UM primer mix (per target)[2]	—	1 µL	1X (UM Primer-80 nM; non-UM Primer-400 nM)
DNA template	—	Variable	

1. Up to four 50X UM Probes can be added to one UM reaction.
2. See Appendix C for details on making a 50X primer mix.

For hydrolysis probes

Reagents	Cat #	Per 50 µL reaction	Final conc.
Nuclease-free water	—	To 50 µL	—
4X Countable PCR Mix	KT0004 (PR0004)	12.5 µL	1X
50X oligo mix[1] (per target)	—	1 µL	1X
DNA template	—	Variable	

1. See Appendix C for details on making a 50X oligo mix.

3. Gently vortex the amplification mix until uniform, briefly centrifuge, and keep at room temperature or 4°C (up to 1 hour) while preparing swing bucket assemblies.

Optional reaction optimization

Magnesium chloride ($MgCl_2$) is a critical cofactor required for DNA polymerase activity and directly impacts PCR efficiency and specificity. The separate 50 mM $MgCl_2$ solution provided in the 4X Countable PCR Mix Kit allows for user-controlled optimization. It should be added during amplification mix preparation. We recommend adding 1 µL or more if the assay requires more specificity against targets but optimization is required on a case-by-case basis, considering amplicon size, primer design, and template complexity.

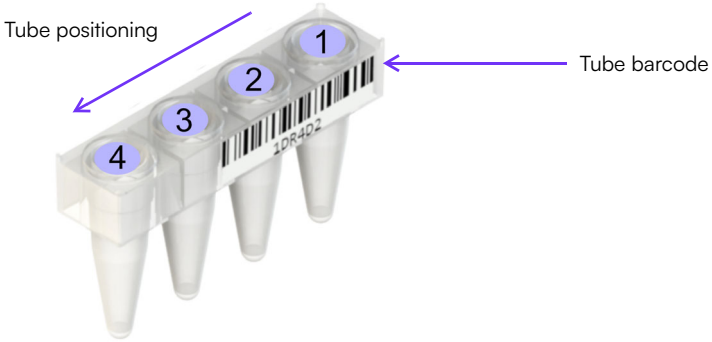
Prepare Countable Swing Bucket assemblies

Note

The Countable System supports high-throughput processing—up to 48 reactions per Countable Swing Bucket Carrier and 192 reactions per kit. This guide is optimized for workflows of 4—8 reactions, but for larger batches, you can scale up with reagent reservoirs and multi-channel pipettes to improve efficiency.

1. Obtain a new Matrix Tube Strip (Cat # KT0001C) from the peel pack and place it in a clean PCR tube rack (not provided) with the **strip barcode facing right**. Label your samples on the **frosted surface opposite the strip barcode**, following the position order shown below to organize experiments.

   Tip: Every Matrix Tube Strip can support up to four PCR reactions of 50 µL each.

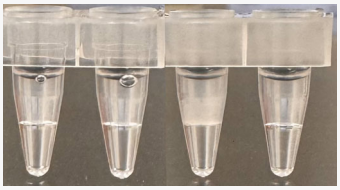


2. Dispense **40 µL** of **MR01 Matrix Reagent** (PR0001) into the bottom of each Matrix Tube Strip. Pipette to the first stop to prevent bubble formation.

   Tip: MR01 Matrix Reagent does not need to be vortexed.

3. Vortex MR02 Matrix Reagent (PR0002) for 2—5 seconds until visually uniform. Gently dispense **90 µL** of MR02 Matrix Reagent to the Matrix Tube Strip on top of MR01 Matrix Reagent to generate a 2-phase solution. MR02 Matrix Reagent should appear slightly opaque. Pipette to the first stop to minimize bubble formation.

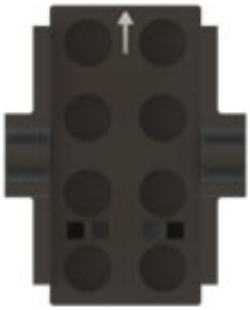
Note

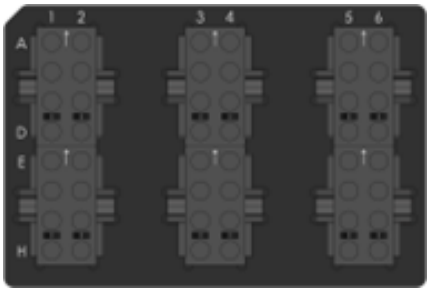


Visually inspect Matrix Tube Strip to confirm a biphasic setup with minimal bubbles. See examples on the left for acceptable appearances.

Do not vortex after this step.

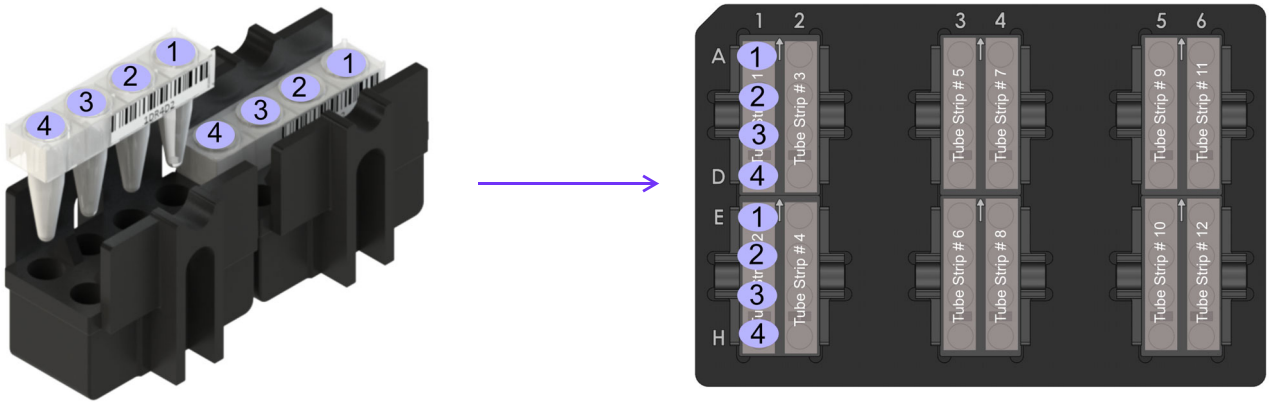
4. Retrieve the Countable Swing Buckets and position them securely in the Countable Swing Bucket Carrier, ensuring they sit flush. Align the carrier so that the notch is in the top left corner.



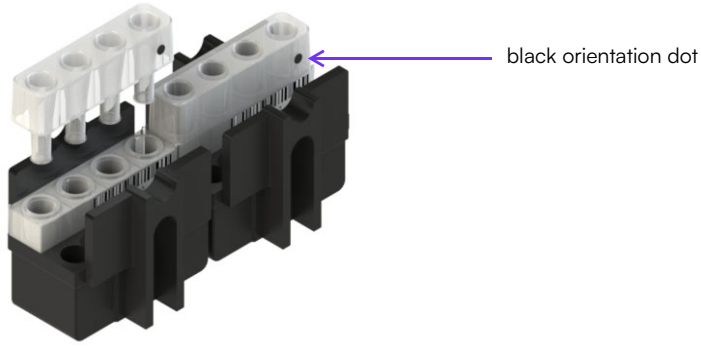


5. Place the Matrix Tube Strips containing MR01 and MR02 Matrix Reagents into the swing bucket with the **barcode side facing right**.

> **Tip:** When using multiple Matrix Tube Strips, load them into the swing buckets in column order to maintain proper tracking.

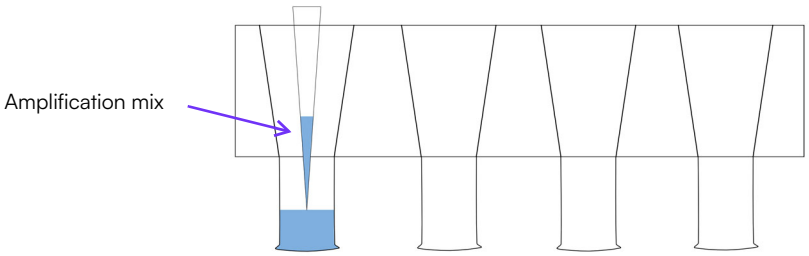


6. Place Matrix Column Strips (Cat # KT0001A) into Matrix Tube Strips with the **black orientation dot** facing right .



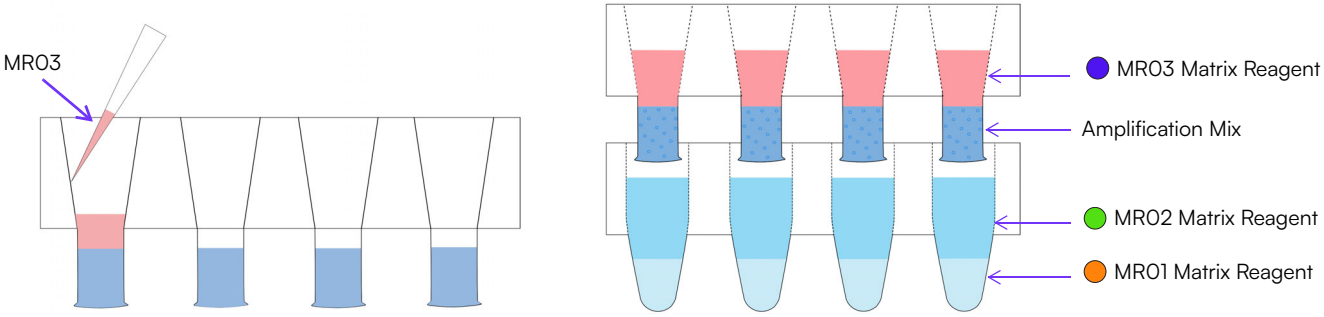
7. Gently add **50 µL** of the amplification mix directly to the bottom of the Matrix Column Strip. Place the pipette tip as close as possible to the Matrix Column Strip membrane without creating a seal (do not pipette at an angle or against the side of the column).

> **CAUTION:** To ensure complete flow-through, the amplification mix must be transferred directly to the bottom of the Matrix Column Strip at a 90° angle. If necessary, lightly touch the membrane to aid proper transfer.



To create water blanks: Gently add **50 µL** of Nuclease-free water directly to the bottom of any **unused** Matrix Column Strips (without amplification mix). Place the pipette tip as close as possible to the Column Strip membrane without creating a seal.

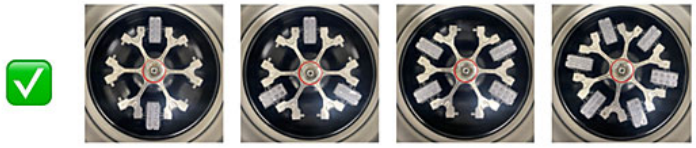
8. Carefully layer 50 µL of MR03 Matrix Reagent (PR0003) on top of the amplification mix by pipetting against the inner wall of the Column Strip (left), ensuring the mix remains undisturbed. Once added, the spin columns are fully assembled (right).



9. Remove a swing bucket from the swing bucket carrier, peel a Matrix Column Strip Seal (Cat # KT0001B) and gently (with light pressure) secure the adhesive side to the top of the Matrix Column Strip to seal the chambers. Remove and discard the end tab. Repeat with all swing bucket assemblies.
10. Proceed **immediately** to centrifugation.

Centrifugation

1. Place a Countable Balance Strip into any swing buckets that has only one Matrix Tube Strip.
2. Place up to **six** swing buckets into a centrifuge, ensuring that the Countable Labs logo "C." is facing the center of the rotor. Confirm proper balance of swing buckets in the centrifuge, and close the rotor lid.



3. Centrifuge for 20 minutes at 16,000 x g at room temperature.
4. Carefully remove each spin column assembly from the swing bucket, and remove the Matrix Column Strip from the Matrix Tube Strip. Visually confirm (see Troubleshooting on page 20) that the amplification mix has successfully flowed through the Matrix Column Strip. Discard the Matrix Column Strip.
5. Place Matrix Tube Strips on a PCR tube rack, and gently seal Matrix Tube Strips with Matrix Tube Strip Caps (Cat # KT0001D).

> **CAUTION:** The matrix is now sensitive to mechanical disruption and must be handled carefully for all following steps. Do not shake, tilt, or agitate the tubes to ensure matrix stability.

6. Proceed to Sample amplification. The Matrix Tube Strips may be stored at 4°C for up to 4 hours.

Sample amplification

1. Evenly distribute the Matrix Tube Strips onto a thermal cycler heat block. Use a Tube Frame (BioRad Cat # 849001) to prevent crushing of the Tube Strips.
2. Perform the thermal cycling protocol with an annealing temperatures appropriate for the specific assay. The default program shown below can be modified based on your specific assay.

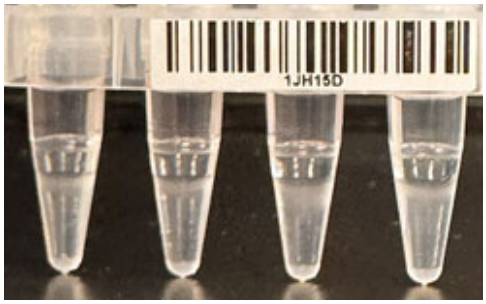
> **Tip:** For the C1000 Touch Thermal Cycler, set the sample volume to 125 µL and heated lid to 105°C.

Ensure ramp rate setting of 2°C/s and adjust annealing temperature as needed per assay.

Cycle	Step	Temperature (°C)	Time
1	Initial denaturation	95°C	20 sec
40	Denaturation	95°C	15 sec
	Annealing & extension	55—60°C[1]	60 sec
1	Hold	4°C	∞

1. We recommend an initial annealing temperature of 55°C when running a new assay for the first time

3. After thermal cycling, three layers should be visible within the matrix. Carefully transfer the Matrix Tube Strips to a clean PCR rack.



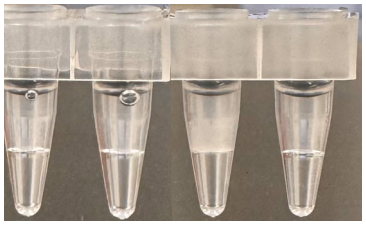
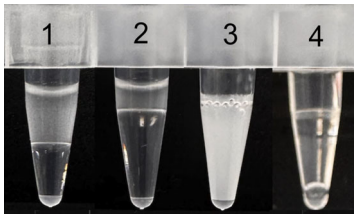
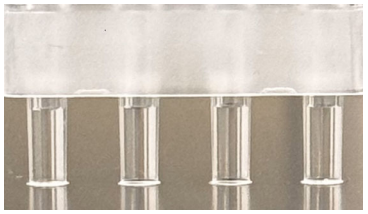
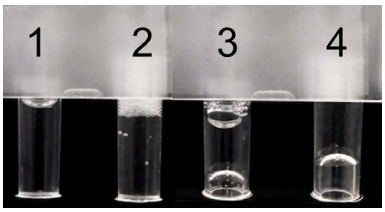
4. Gently wipe the external walls of each Matrix Tube Strip with a lint free wipe to remove any residue or debris prior to loading into the Countable System carousel.
5. Proceed to imaging the samples on the Countable System. Refer to the *Countable System Instructions For Use* (IFU003).

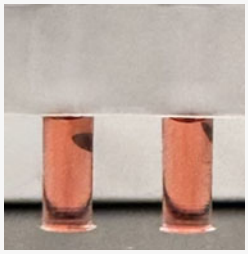
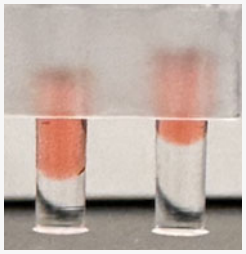
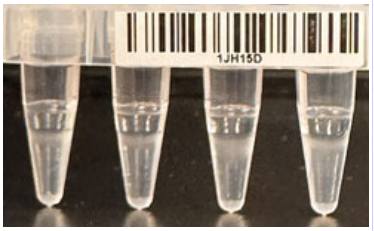
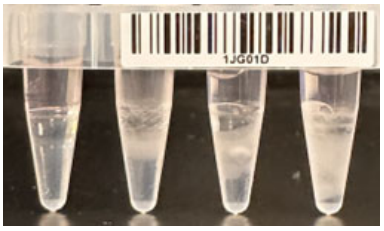
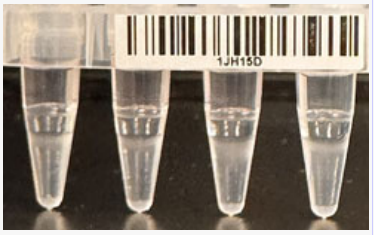
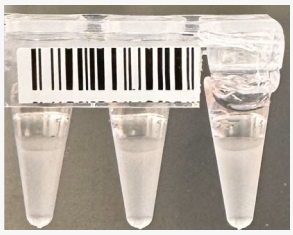
> **Tip:** For more sensitive applications, we recommend imaging the samples as soon as possible after amplification. For other applications, you may choose to store amplified samples for up to 24 hours at 4°C before proceeding to imaging.

CHAPTER 4

Troubleshooting

If any of the following failures occur during the workflow, do not proceed to imaging. We recommend preparing the sample again, following the appropriate mitigation procedures. Contact technical support at success@countablelabs.com for further assistance.

Step	Normal	Incorrect	Mitigation
Countable Swing Bucket assemblies	 Biphasic setup has small bubbles that do not disturb the layers (all are acceptable).	 Tube 1 shows correct depiction. Tubes 2-4 show incorrect Matrix Reagent volumes, non-distinct layers, or a bubble at the bottom of the tube.	Pipette slowly and only to the first stop to prevent bubble formation.
	 Amplification mix is input directly to the bottom of all columns.	 Tube 1 shows correct depiction. Tubes 2-4 show bubbles at the top or bottom of the amplification mix volume.	Amplification mix must be transferred directly to the bottom at a 90° angle of the Column Strip. If necessary, lightly touch the membrane to aid in proper transfer.

Step	Normal	Incorrect	Mitigation
Spin	 Complete flow-through after centrifugation. Only MR03 Matrix Reagent (pink) is remaining in the column after spin, indicating successful matrix generation.	 Incomplete flow-through after centrifugation. Amplification mix (clear layer) appears below the MR03 Matrix Reagent (pink) indicating an error in matrix generation.	This issue may be caused by physical impurities in DNA samples or oligo mixtures. Purify samples and/or oligos using recommended filtration methods or cleanup kits prior to use.
Centrifugation and sample amplification	 Three clear (but distinct) layers are seen in the Matrix Tubes after centrifugation and amplification.	 Layers are disrupted or not distinct after centrifugation and amplification, indicating that matrix stability has been compromised.	Verify the amplification mix was prepared according to the protocol. Ensure the correct amount of MR01 and MR02 are pipetted. Ensure minimal mechanical disruption during handling. Check DNA samples for potential contaminants (e.g., detergents or residual solvents) that may interfere with amplification.
Sample amplification	 Tubes are all the same size and shape after amplification.	 Tube 1 is crushed from the thermal cycler, indicating that the PCR tube frame was not properly used.	Confirm that the PCR tube frame was correctly positioned and used during the PCR amplification step. This ensures proper thermal contact and reaction consistency.

Appendices

Appendix A: Experimental controls

Negative controls

Establishing a baseline signal is essential for accurate single-molecule detection. We recommend the use of both no template controls (NTCs) and negative controls (NCs) to monitor for non-specific amplification and assay noise.

- **No template controls (NTCs):** Prepared identically to test samples but without added DNA template. Used to detect contamination, primer-dimer formation, or nonspecific amplification.
- **Negative controls (NCs):** Contain background DNA at concentrations similar to test samples but lack the target of interest. Used to assess false-positive molecule detection.

Multiple replicates of NTCs and NCs are recommended to determine assay-specific background levels. The false positive threshold is typically defined as two standard deviations above the average background count.

For high-specificity applications, we recommend using HPLC-purified oligos and probes to reduce synthesis-related artifacts that may contribute to non-specific signal.

Regular monitoring of negative controls supports assay optimization and helps assess reagent stability and workflow consistency over time.

Training samples

In Countable PCR, training samples function as analysis positive controls within the Countable Control Software of the Countable System. It is intended to be run for every experiment. Training samples enable run-specific algorithm calibration, improving classification of true single-molecule signals and increasing confidence in low-count detection.

This calibration enhances the specificity of single-molecule detection, particularly in low-abundance samples (<1,000 counts per 50-µL reaction), where statistical noise becomes an issue and where single-molecule specificity is critical for many applications.

A training sample should closely resemble your test samples in both DNA size and input amount, as these parameters can influence signal quality and assay performance.

To optimize Countable PCR performance, we recommend to:

- Include at least one training sample per channel per run
- Ensure the sample contains 10,000—100,000 counts per 50-µL reaction
- Use synthetic spike-ins when necessary
- Include all probes and oligos present in the experimental panel to support channel-specific calibration

In multiplex assays, a single training sample can serve as a positive control across all detection channels, provided sufficient target counts are present in each.

Appendix B: Universal Multiplex sequences

The following table contains the sequence for each Universal Multiplex Probe (Cat # KT0005) that should be appended to the 5' end of a primer to construct an UM primer.

UM probe name	5’ Appending sequence
50X UM-1 Probe	5'-TAGAAGGCACAGTCGAGG-3'
50X UM-2 Probe	5'-CAGAAGACGGCATACGAGAT-3'
50X UM-3 Probe	5'-ACCGTAGAGTCCGAGCAA-3'
50X UM-4 Probe	5'-GAAGCGTTTATGCGGAAGAG-3'

Appendix C: Fluorophore compatibility of hydrolysis probes

The Countable System supports up to 4 fluorescence channels, with commonly used fluorophores listed below. Some fluorophores yield higher signal-to-noise ratios due to brightness and spectral properties. Our fluorophore recommendations reflect optimal performance based on our internal testing.

Countable System channel	Compatible 5' fluorophore	Recommend fluorophore for best performance
Ch01	FAM, Alexa488, Atto488	Alexa488
Ch02	HEX, VIC, Atto532	Atto532
Ch03	TEX615, Atto590, Alexa594	Alexa594
Ch04	Cy5, Atto647N	Atto647N

Appendix D: Probe additive design

For hydrolysis probe (HP)-based assays, you can use probe additives (PAs) to improve quenching efficiency and reduce background fluorescence.

Following are basic design guidelines:

1. Truncate the HP sequence from the 3’ end to generate a 10—14 bp fragment, retaining the 5’ sequence.
2. Estimate melting temperature (Tm) using an oligo analysis tool (e.g., IDT OligoAnalyzer).
3. Select a truncated sequence with a Tm of 35—45°C.
4. Generate the reverse complement of the truncated sequence.
5. Append the appropriate 3’ quencher, matched to the 5’ fluorophore.

PA design example

Probe sequence	/56-FAM/CTAGCCAGAGACATCAAGAAT/3IABkFQ/
Truncated probe	5'-CTAGCCAGAGA-3' (Tm = 41.6°C)
Reverse complement	5'-TCTCTGGCTAG-3'
PA sequence	5'-TCTCTGGCTAG/3IABkFQ/

For questions or assistance with probe additive design, contact technical support at success@countablelabs.com.

Appendix E: Preparing 50X oligonucleotide mixes

Use the tables below to prepare concentrated 50X oligo mixes for either Universal Multiplexing or hydrolysis probe assays. You can prepare and store the mixes in advance according to conventional oligo stability guidelines.

For every reagent below, prepare a 100 µM stock in TE buffer.

50X primer mix for Universal Multiplex per target

Reagents	Concentration in 50X (µM)
UM primer	4
Non-UM primer	20

50X Oligo mix for hydrolysis probes per target

Reagents	Concentration in 50X (µM)
Forward primer	10
Reverse primer	10
Hydrolysis probe	5
Probe additive (PA)	15

Appendix F: Quick start guide

01 Prepare amplification mix

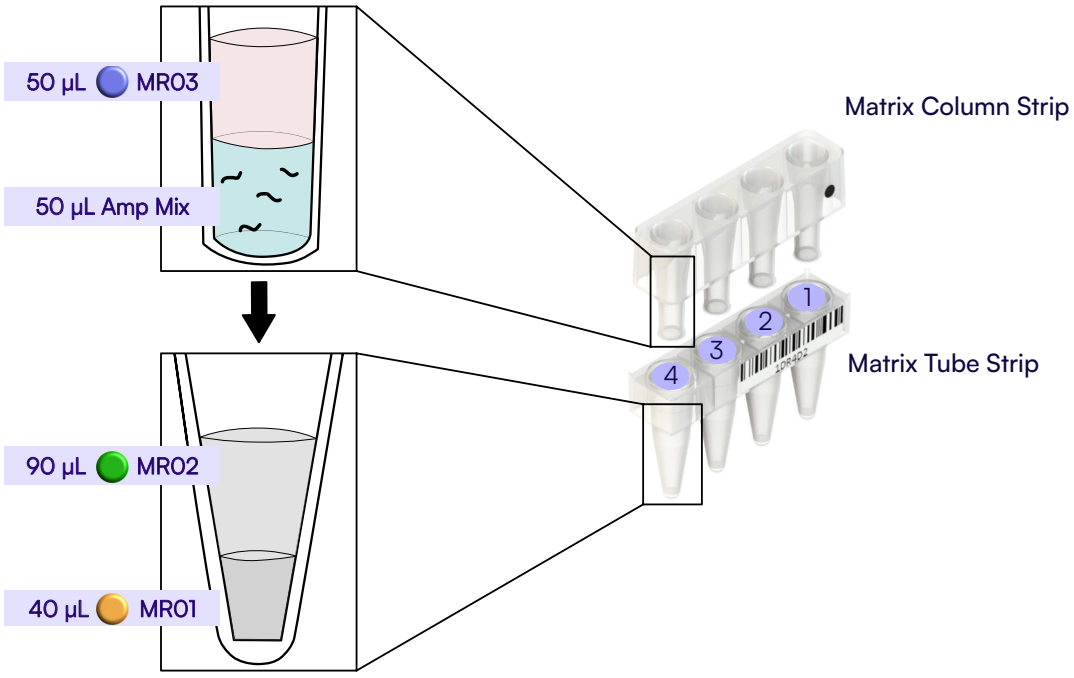
For Universal Multiplexing

Reagent	Cat #	Per 50 µL reaction
Nuclease-free water	-	To 50 µL
4X Countable PCR Mix	KT0004 (PR0004)	12.5 µL
50X UM 1-4 Probes	KT0005 (PR0006-PR0009)	1 µL/probe
50X UM primer mix (per target)	-	1 µL
DNA template	-	Variable

For hydrolysis probes

Reagent	Cat #	Per 50 µL reaction
Nuclease-free water	-	To 50 µL
4X Countable PCR Mix	KT0004 (PR0004)	12.5 µL
50X oligo mix (per target)	-	1 µL/target
DNA template	-	Variable

02 Column Strip assembly



03 Spin at 16,000 x g for 20 minutes at RT

04 Amplify in a recommended thermal cycler using the following protocol:

Cycle	Step	Temp	Time
01	Initial denaturation	95°C	20 s
40	Denaturation	95°C	15 s
	Annealing & extension	55-60°C	60 s
01	Hold	4°C	∞
Sample volume: 125 µL	Lid temperature: 105°C	Ramp rate: 2°C /s	

05 Proceed to imaging the samples on the Countable System

Countable Labs.

Countable Labs, Inc.
1810 Embarcadero Rd.
Suite 200
Palo Alto, CA 94303

IFU004 Rev 1.0

For Research Use Only. Not for Use in Diagnostic Procedures.

countablelabs.com