Technical note:

Increase sample throughput and identify multiplets using BD™ Single-Cell Multiplexing Kit

Overview

The BD™ Single-Cell Multiplexing Kit utilizes oligonucleotide-conjugated antibodies to provide a high sample throughput for single-cell 3' RNA-seq assays. A set of 12 antibodies in the kit target the same universally expressed cell-surface antigen. Each antibody is conjugated with a Sample Tag, a unique 45-nucleotide barcode sequence **(Figure 1)**. Adjacent to the **Sample Tag** barcode, a universal PCR handle and poly(A) tail allows each Sample Tag to be captured by oligo-dT beads, such as the BD Rhapsody Cell-Capture Bead, and then amplified by PCR using 2 Sample-Tag specific primers targeting the PCR handle region, along with BD Rhapsody reagents.

Figure 1. Structure of a Sample Tag and its compatibility for capture by BD Rhapsody Cell-Capture Bead. UMI – unique molecular identifier; CL – cell-label barcode.

Following a simple antibody staining of Sample Tags to single-cell suspension, multiple samples can be combined to perform a single-sample library preparation (**Figure 2**, also see *BD Single-Cell Multiplexing Kit – Human Protocol Doc ID: 54478*). Sample origin is identified after sequencing using the sample determination algorithm (see *BD Single-Cell Genomics Analysis Setup User Guide Doc ID: 47383 Rev 2.0,* and *BD Single-Cell Genomics Bioinformatics Handbook Doc ID: 54169 Rev 2.0*). The ability to tag and pool multiple samples into a single cartridge greatly enhances sample throughput and flexibility in experimental design.

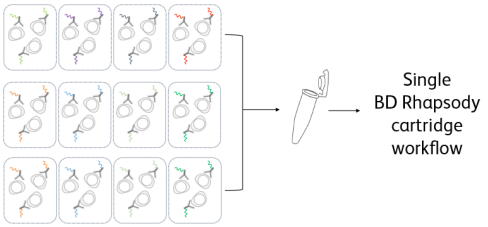

Figure 2.
BD Single-Cell Multiplexing Kit allows up to 12 samples to be pooled together in a single cartridge workflow for BD Rhapsody.

Detection of cross-sample multiplets with the BD Single-Cell Multiplexing Kit

In addition to high sample throughput and low library preparation costs, the BD Single-Cell Multiplexing Kit enables users to detect cross-sample **multiplets**. Multiplets are derived from events where more than one cell is captured by BD Rhapsody Cell-Capture Bead **(Figure 3)**. The occurrence of multiplets increases as more cells are loaded into the BD Rhapsody cartridge, following Poisson distribution calculations **(Table 1)**. Even though the BD Rhapsody microwell technology provides low multiplet occurrence compared to conventional droplet-based technologies, the user still makes a decision to limit the number of cells loaded into a cartridge to maintain a low multiplet rate. In some cases, multiplets are not easily identifiable, and can be misinterpreted as biologically meaningful.

To understand more about multiplets, consider a simple example in which a pool of 1000 Ramos cells labeled with Sample Tag 3 and 1000 BT549 cells labeled with Sample Tag 4 were loaded onto a single BD Rhapsody cartridge.

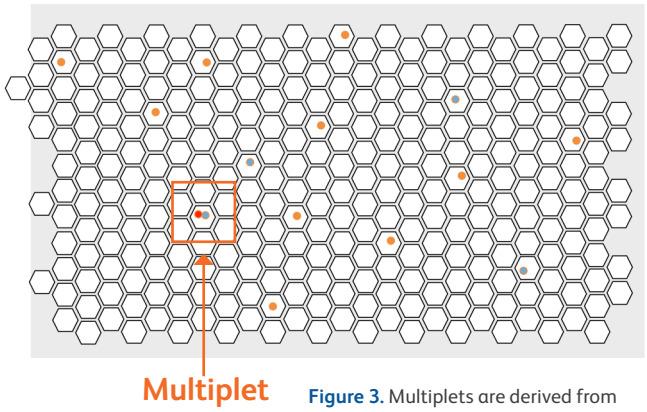

Figure 3. Multiplets are derived from >1 cell entering a microwell and may skew single-cell gene expression analysis.

Single-cell gene expression profiles were visualized using tSNE **(Figure 4)**. In addition to BT549 and Ramos clusters, a small cluster was observed in between the main clusters **(Figure 4A)** and was identified as cross-sample multiplet by the Sample Determination algorithm, by the presence of both sample tags in the same cell. The identity of the multiplet cluster is further supported by the co-expression of BT549 and Ramos markers. For instance, BT549 marker LGALS1 and Ramos marker CD79A **(Figure 4B-C)**.

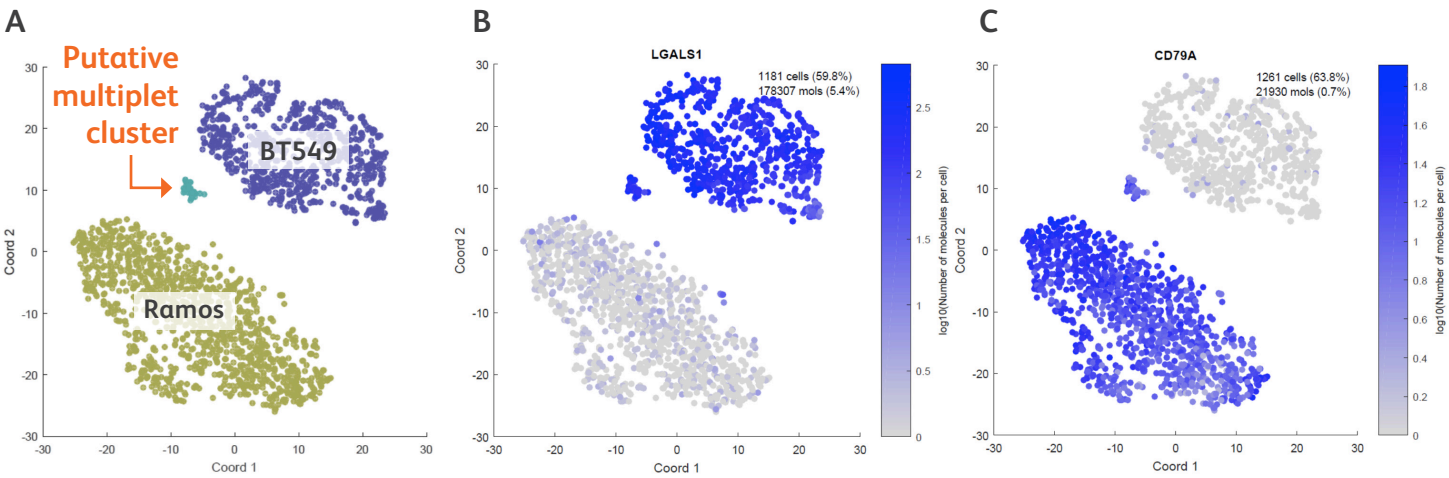

Figure 4. 1000 Ramos cells labeled with Sample Tag 3 and 1000 BT549 cells labeled with Sample Tag 4 were pooled and analyzed on a single BD Rhapsody cartridge.

# of cells loaded in BD Rhapsody cartridge	% total theoretical multiplets	% theoretical multiplets that remain after identification with N Sample Tags*		
		N=2	N=4	N=8
1,000	0.24%	0.12%	0.06%	0.03%
5,000	1.19%	0.59%	0.30%	0.15%
10,000	2.36%	1.19%	0.59%	0.30%
15,000	3.53%	1.78%	0.89%	0.45%
20,000	4.69%	2.36%	1.19%	0.59%

Table 1. Theoretical percentage of multiplets increases as more cells are loaded into both a competitor's droplet based system and the BD Rhapsody cartridge. Adding Sample Tags, even for the same single-cell sample, can identify inter-sample multiplets as distinguished by different Sample Tags. Multiplets that are formed by cells with the same Sample Tag cannot be detected by the pipeline. Note: Though it is not validated, users can load more than 20,000 cells per cartridge and continue to remove multiplets in data analysis.

*Calculations are based on equal number of cells per Sample Tag added to the BD Rhapsody cartridge.

Increase cell number analyzed while confidently identifying multiplets

An advantage of using the BD Single-Cell Multiplexing Kit is that one can load a higher number of cells while maintaining a low rate of unidentified multiplets. To illustrate this example, a dataset of 4 sample types—peripheral blood mononuclear cells (PBMCs), Ramos B cells, Jurkat T cells, and T47D breast cancer cells—split across 12 Sample Tags **(Figure 5)** were used to demonstrate the assay's ability to identify multiplets between cell type **(Figure 6)** and of the same cell type **(Figure 7)**.

In this 20,000 cell-load experiment, the theoretical multiplet occurrence was ~4.7%; using Sample Tags, 4.3% of the putative cells were identified as multiplets **(Figure 6A-B, Table 1)**. When gene expression profiles were projected using tSNE and overlaid with the Sample Tag identity, many of the multiplets identified by the Sample Tag determination algorithm resided in small clusters between the major cell populations **(Figure 6A)**. These small clusters expressed gene markers from more than one cell type (not shown in this Technical Note), thereby validating the use of Sample Tags for multiplet identification.

The BD Single-Cell Multiplexing Kit also can be incorporated into a single-cell suspension sample to identify multiplets. In this use case, instead of staining Sample Tags per sample, a single sample can be split into multiplet Sample Tags. An example is shown in the 12-Sample Tag experiment where each sample type was split between 3 Sample Tags and pooled at an even ratio **(Figure 6)**. By visualizing a single cell type only (Jurkat cluster in **Figure 7A**), the cross-sample multiplets were identified by the Sample Determination algorithm even within the same cell type. Many of these cross-sample multiplets had higher gene detection **(Figure 7B)** and more mRNA molecules detected **(Figure 7C)**, suggesting that these were true multiplets. The BD Single-Cell Multiplexing kit can be used to identify multiplets even within a single sample.

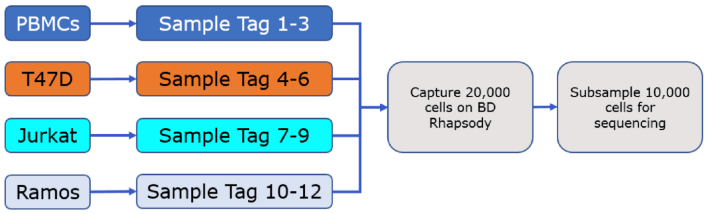

Figure 5. Schematic of the 12 Sample Tag experiment with 20,000 cells of mixed sample types loaded on BD Rhapsody.

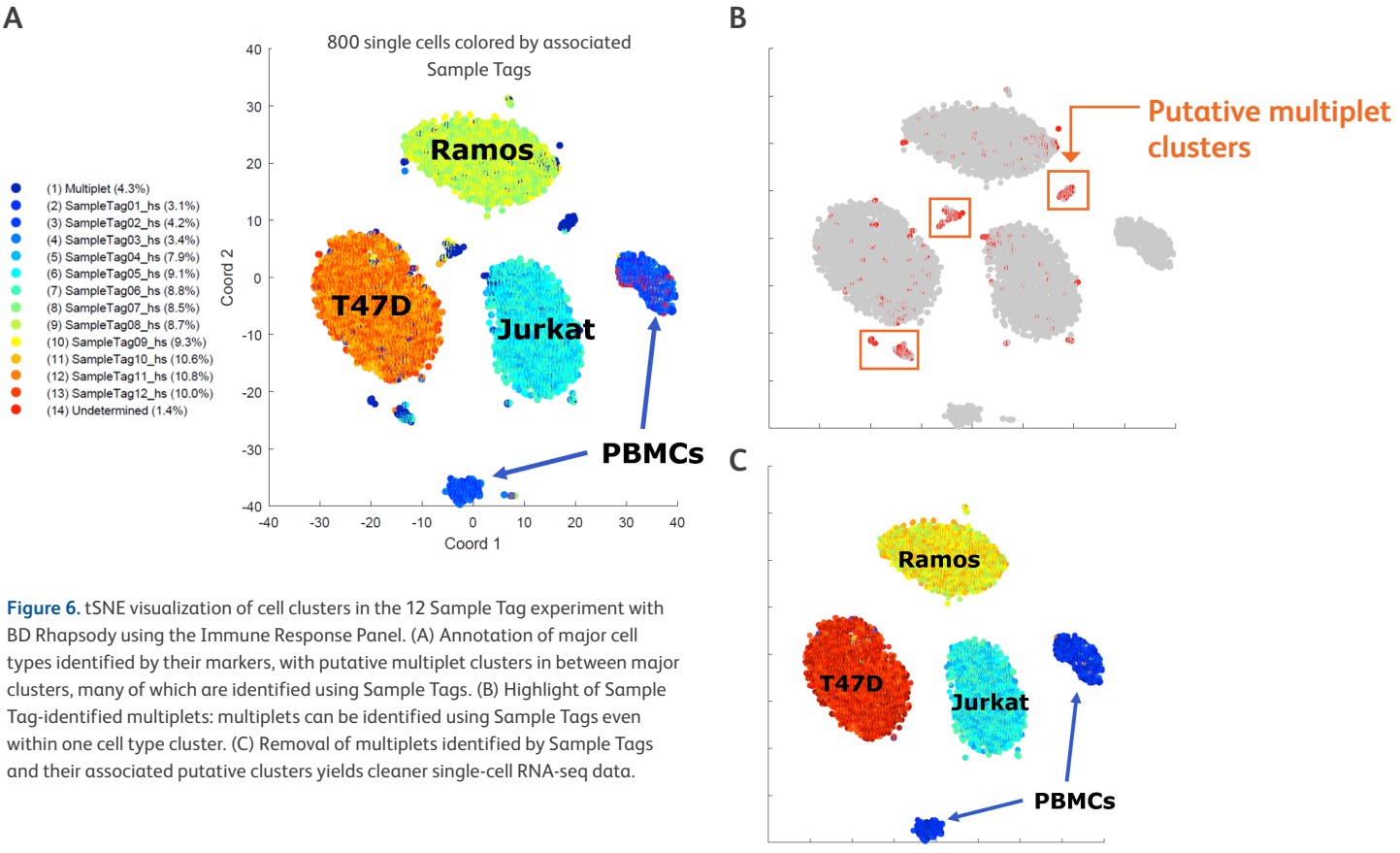

Figure 6. tSNE visualization of cell clusters in the 12 Sample Tag experiment with BD Rhapsody using the Immune Response Panel. (A) Annotation of major cell types identified by their markers, with putative multiplet clusters in between major clusters, many of which are identified using Sample Tags. (B) Highlight of Sample Tag-identified multiplets: multiplets can be identified using Sample Tags even within one cell type cluster. (C) Removal of multiplets identified by Sample Tags and their associated putative clusters yields cleaner single-cell RNA-seq data.

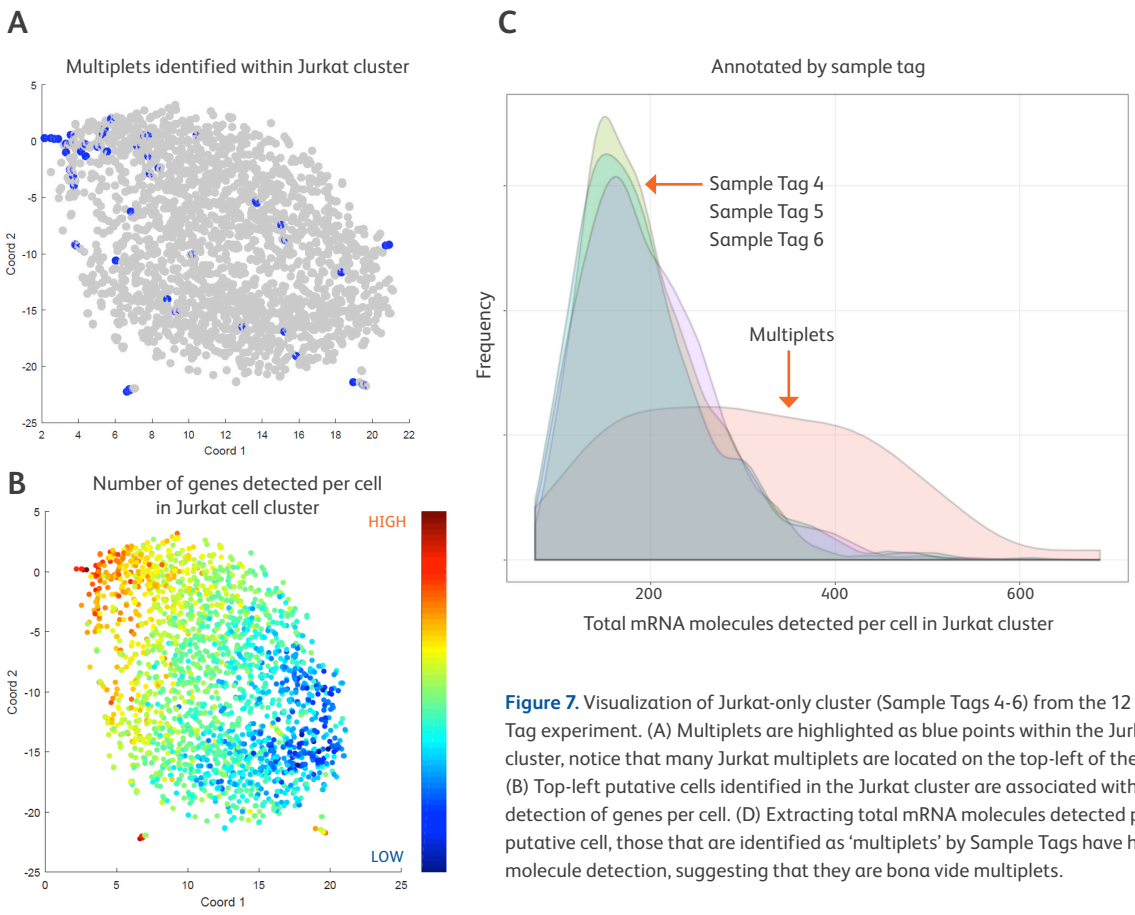

Figure 7. Visualization of Jurkat-only cluster (Sample Tags 4-6) from the 12 Sample Tag experiment. (A) Multiplets are highlighted as blue points within the Jurkat cluster, notice that many Jurkat multiplets are located on the top-left of the cluster. (B) Top-left putative cells identified in the Jurkat cluster are associated with higher detection of genes per cell. (D) Extracting total mRNA molecules detected per putative cell, those that are identified as 'multiplets' by Sample Tags have higher molecule detection, suggesting that they are bona vide multiplets.

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