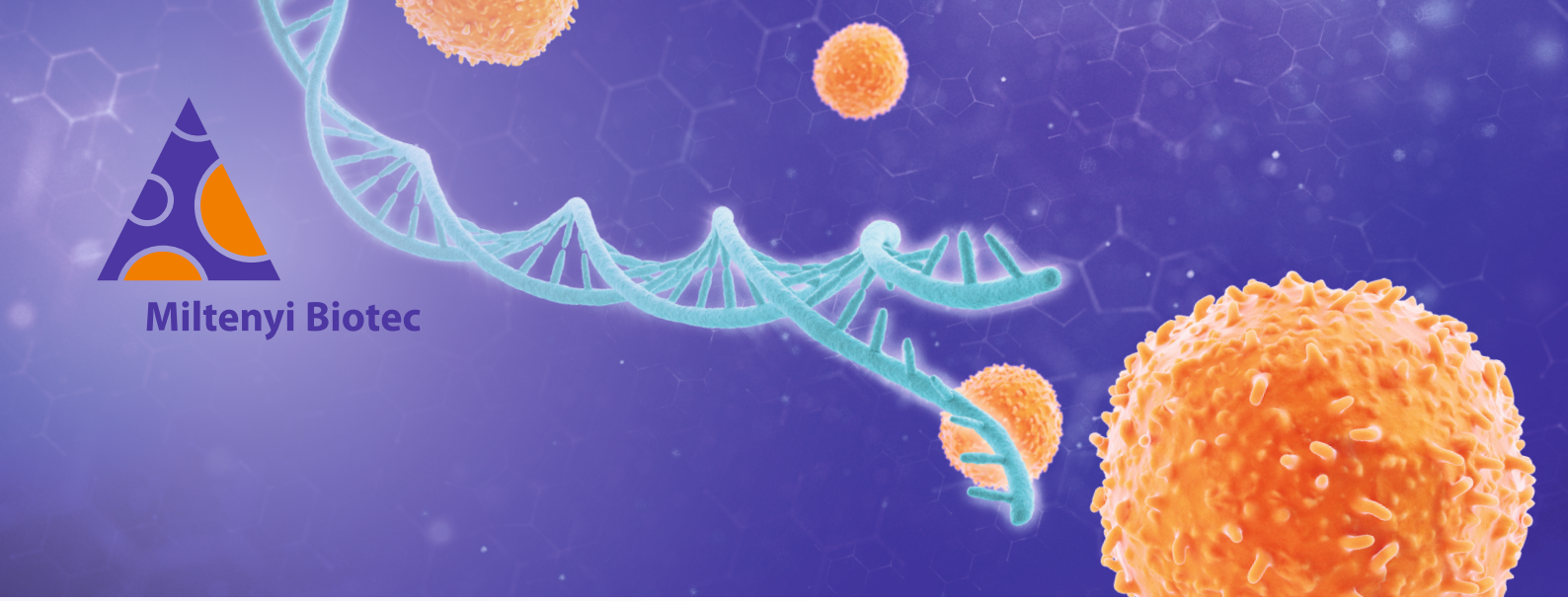

Cleaner samples, clearer data

A one-step sample cleanup workflow for high-quality single-cell RNA sequencing

Sample preparation is fundamental to the success of any single-cell RNA sequencing (scRNA-seq) experiment. It determines the quality and reliability of downstream data and thus the success of the experiment. In addition, well-prepared samples can have a major impact on the reproducibility, reliability, and actual cost of the whole experiment, while high technical noise due to poor sample preparation can affect data interpretation and, in the worst case, lead to unwanted misinterpretations and false conclusions. The solution to prevent this is to effectively remove dead cells, red blood cells (RBCs), and debris. Here, we describe a one-step workflow that combines several important cleaning steps into a single step, significantly speeding up the sample cleaning process while maintaining high sample quality without negatively impacting the transcriptome.

This application note demonstrates the effectiveness of the Cell Suspension Cleanup Kit, human (CSCK)*, on fresh and cryopreserved human high-grade serous ovarian carcinoma (HGSOC) samples that were dissociated with the gentleMACS™ Technology using the Tumor Dissociation Kit, human. The quality of the scRNA-seq data was thus improved, as demonstrated by several quality control (QC) parameters. These include median gene or UMI counts per cell and reduced cell-free content, such as mitochondrial reads. We also compared this one-step CSCK workflow with a commercially available two-step alternative. Using whole exome RNA sequencing data, we identified significant transcriptional changes. In short, the one-step method not only resulted in higher viability and improved input material for high-quality scRNA-seq data, but also reduced susceptibility to transcriptional alterations during the cleanup process.

Materials and methods

Sample origin information

HGSOC samples were kindly provided by Evangelisches Krankenhaus Frauenklinik Bergisch Gladbach, Germany (Prof. Dr. Rudlowski). All patients included in this study gave informed consent (ethical vote medical association Nordrhein: 2024026). Whole blood samples were withdrawn from healthy donors who gave informed consent (ethical vote medical association Nordrhein: 2020272).

Long-term storage of tumor samples

HGSOC samples were cryopreserved as tissue pieces in MACS® Freezing Solution and stored in liquid nitrogen for 6 to 17 months for later bulk RNA-seq experiment.

Tumor dissociation

For scRNA-seq: Freshly obtained HGSOC samples were dissociated immediately after acquisition using the gentleMACS Octo Dissociator with Heaters and the Tumor Dissociation Kit, human, in combination with gentleMACS C Tubes. After tissue dissociation, single-cell suspensions were filtered through MACS SmartStrainers (70 µm) and further processed for cleanup and scRNA-seq.

For bulk RNA sequencing: Cryopreserved tumor samples were thawed and dissociated using the gentleMACS Octo Dissociator with Heaters and the Tumor Dissociation Kit, human, in combination with gentleMACS C Tubes. After tissue dissociation, single-cell suspensions were filtered through MACS SmartStrainers (70 µm). To measure standard QC parameters prior to sequencing experiments, an aliquot of cells was used for flow cytometry on the MACSQuant® 10 Analyzer and Countess™ 3 FL Automated Cell Counter (Thermo Fisher Scientific®).

* Please note, the Cell Suspension Cleanup Kit, human, is not compatible with digestion enzymes degrading glycopherin A, including the following Miltenyi Biotec Kits: Brain Tumor Dissociation Kit (P), human (130-095-942); Umbilical Cord Dissociation Kit, human (130-105-737); Embryoid Body Dissociation Kit, human and mouse (130-096-348); Whole Skin Dissociation Kit, human (130-101-540); Multi Tissue Dissociation Kit 2 (130-110-203). As a workaround for these samples, you can deplete RBCs using the Red Blood Cell Lysis Solution and subsequently use either the Cell Suspension Cleanup Kit, human, to deplete dead cell and organelles, or, if only dead cells shall be removed, the Dead Cell Removal Kit.

Sample cleanup
For scRNA-seq: After dissociation, single-cell suspensions were divided into two fractions. One fraction was the non-processed origin fraction (Ori) and the second one was purified using the workflow with CSCK.

For bulk RNA sequencing: After dissociation, whole blood was added to the single-cell suspension for all samples to compensate for the loss of erythrocytes during the freeze-thaw cycle, after which the sample was divided into three fractions. RNA of one unprocessed fraction was immediately isolated and was later compared to the second and third fraction. The second fraction was processed using the one-step cleanup workflow with CSCK. The third fraction was cleaned up using a two-step workflow with commercially available products, including a column-free dead cell removal solution and a red blood cell lysis solution. All samples were processed according to the manufacturer's instructions.

Flow cytometry analysis
After treatment, all samples were stained for a viability check. Three samples were stained for testing erythrocyte and mitochondrial content using anti-human antibody conjugates manufactured by Miltenyi Biotec.

The following reagents were used:
CD45-VioGreen™, Annexin V-FITC Kit, CD326 (EpCAM)-PE, CD31-PE-Vio® 770, CD90-APC, CD235a(GlyA)-APC Vio 770, DAPI, and propidium iodide (PI).

Flow cytometry analysis was performed using the MACSQuant Analyzer 10.

Measurement of staining efficiency
In parallel to flow analysis, a sample aliquot was used to validate sample count, viability, and staining efficiency on the Countess 3 FL Automated Cell Counter using ReadyCount™ Green/Red Viability Stain following manufacturer's instructions (Thermo Fisher Scientific). For optimal NGS results, the research community commonly recommends a staining efficiency of 70–80%. Furthermore, 10× Genomics® recommends a maximum RBC content of 20% in the sample.[1]

scRNA-seq methods
Single-cell suspensions processed with CSCK and unprocessed samples as control from three freshly obtained HGSOC samples were resuspended in PBS with 0.04% non-acetylated BSA. They were then subsequently processed using the Chromium™ Next GEM Single Cell 5' Reagent Kit (Dual Index) according to the manufacturer's instructions (10× Genomics).
CSCK-processed and unprocessed samples from each tumor donor were sequenced to a depth of >20,000 reads per cell using a NextSeq® 2000 Sequencing System (Illumina®). Sequencing data were processed using the Cell Ranger software and the results were visualized using the Loupe Cell Browser software (both 10x Genomics).

Whole transcriptome analysis
RNA was isolated from three tumor samples using the RNeasy® Kit (QIAGEN®) and quantified using Qubit™ 4 Fluorometer (Thermo Fisher Scientific). Sample quality was determined with an automated electrophoresis tool using the 2100 Bioanalyzer® Instrument (Agilent Technologies). The following library preparation was performed using QIAseq™ Stranded mRNA Library Kit and HiFi PCR Master Mix (QIAGEN). The library was sequenced on the NextSeq 2000 sequencer. Transcriptome sequencing data analysis was performed via CLC Genomics Workbench/Server version 22.0 (QIAGEN) covering trimming of reads, read mapping (hg38, forward strand only), differential gene expression analysis ('While controlling for = Donor'), and principal component analysis. Genes with a false discovery rate of 1.5 were considered to be differentially expressed.

Results

One-step quality improvement
Single-cell suspensions were prepared from five freshly obtained HGSOC samples following the process described in the materials and methods section and also shown in figure 1.

Figure 1: Schematic representation of the sample processing workflow. Samples can be stored or transported in MACS Tissue Storage Solution before undergoing tissue dissociation with the gentleMACS Octo Dissociator with Heaters. The resulting single-cell suspension is subjected to a one-step magnetic cleanup with CSCK to remove debris, dead cells, red blood cells (RBCs), and cell-free organelles. This ensures high-quality input for downstream applications such as bulk or scRNA-seq.

Quality parameters were assessed using the MACSQuant 10 Analyzer and Countess 3 FL in conjunction with the ReadyCount Green/Red Viability Stain. The proportion of dead cells was analyzed using the viability marker PI and RBCs using GlyA antibodies. Tom22 was selected as a marker for mitochondria, which were determined to be representative of cell-free organelles (fig. 2). The enriched sample (CSCK) was compared to the original unpurified fraction (Ori) immediately after dissociation.

With CSCK, all relevant quality parameters were increased after the one-step magnetic separation (cleanup) run compared to the untreated sample. Viability was increased from 52% to 77% (fig. 2A), while RBC and cell-free organelle content was reduced to 6.8% and 7.4%, respectively (fig. 2B and 2C). This ultimately resulted in an increase in overall staining efficiency measured with the Countess 3 FL from 37% to 89% (fig. 2D), representing the percentage of viable nucleated cells. Importantly, sample cleanup with CSCK resulted in compliance with the thresholds, indicating that the sample was well prepared. Moreover, the staining efficiency value of the automated cell counter is a decisive factor in determining the suitability for the subsequent sequencing. The higher the value, the more predictive it is of sequencing success.

Cleanedup samples improve scRNA-seq results

scRNA-seq of single-cell suspensions from three of the five freshly obtained HGSOC samples was performed on unprocessed and purified cells using CSCK. Various NGS quality parameters were plotted to highlight the effect on data reliability when a sample is properly prepared. The proportion of valid barcodes differed between the samples cleaned with CSCK and the unprocessed samples, with higher values in the cleaned sample resulting in a better match to the barcode whitelist. The unprocessed sample had more sequencing errors, reducing the proportion of valid barcodes.

Next, the reads that were confidently mapped to intergenic and transcriptomic regions were analyzed (figs. 3B and C). While fewer intergenic regions were detected, overall a much higher proportion of reads were confidently mapped to the transcriptome in the CSCK-processed sample. This indicates more relevant data generated in the scRNA-seq experiment after CSCK purification compared to the control (Ori).

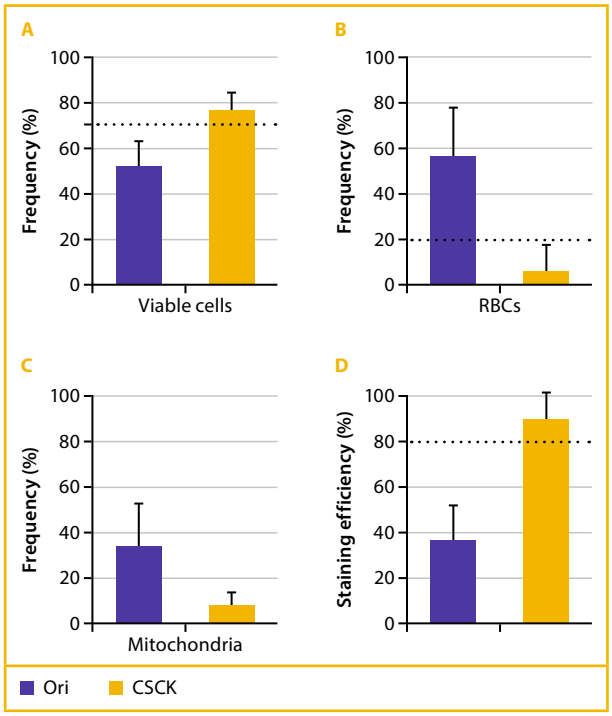

Figure 2: Cleanup performance of freshly obtained HGSOC samples: (A) Viability before and after processing the sample with CSCK. (B) Frequency shows the frequency of RBCs with a threshold set at 20%, as recommended for single-cell RNA sequencing[1], and (C) the frequency of cell-free organelles (mitochondria). (D) Staining efficiency, which is a critical factor for subsequent successful single-cell RNA sequencing. Dotted lines represent the respective threshold of what has been commonly used as a quality indication for viability, RBC content, and staining efficacy (n = 5).

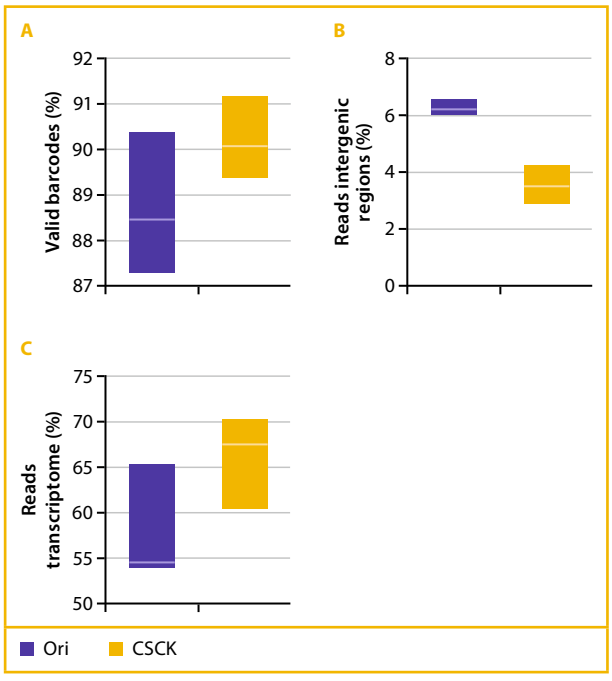

Figure 3: Optimizing sequencing results of freshly obtained HGSOC samples. scRNA-seq quality metrics comparing unprocessed (Ori) and samples cleaned with CSCK. (A) Distribution of valid barcodes. (B) Distribution of reads mapped confidently to intergenic (non-coding) regions, and (C) proportion of reads mapped confidently to the transcriptome (n = 3).

Removal of RBCs and cell-free organelles increases readout quality

In addition to dead cells, the CSCK also effectively removes RBCs and cell-free organelles, like mitochondria and cell-free nuclei, which can have a huge impact on the quality of the sequencing data. To demonstrate this, scRNA-seq was performed with unprocessed (Ori) and CSCK-processed freshly obtained HGSOC samples. Figure 4A presents a representative result from a CSCK-processed sample showing a UMAP projection with general cell type annotation. Resulting cell populations exhibit clear separation, enabling detailed downstream analysis, including the identification and investigation of rare cell subsets. Additionally, RBC-associated gene expression (HBB^+) was analyzed, and the number of positive reads was compared between the Ori and CSCK (fig. 4B). A significantly higher number of HBB^+ reads were detected in the unprocessed (Ori) sample, potentially confounding cell population assignments and leading to misinterpretations. CSCK-processed samples, show a strong reduction of these reads. Another crucial factor of technical noise can be caused by a high mitochondrial (mt) read count. Figure 4C shows that CSCK was able to significantly reduce this amount compared to the unprocessed sample, confirming the result shown in figure 2C.

Reducing unwanted sequencing reads enables a lower read threshold, allowing for a more detailed and comprehensive view of the data, such as the analysis of rare cell populations.

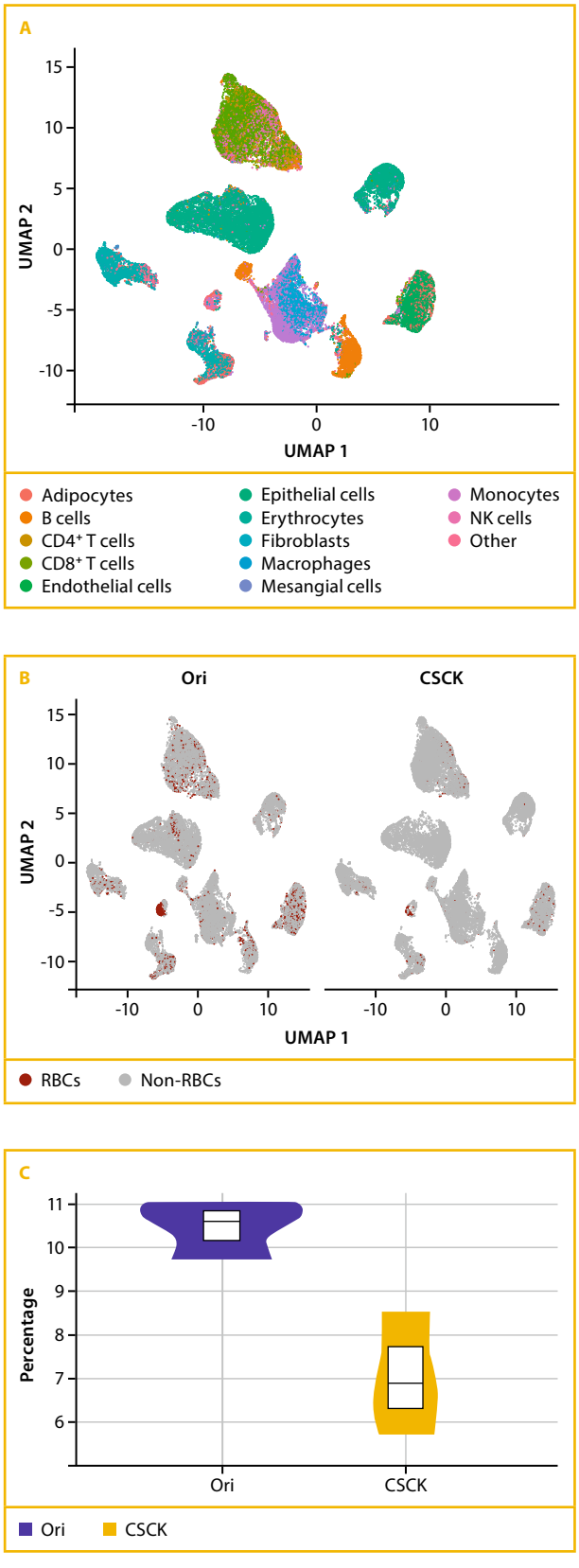

Figure 4: Enhancing output quality and reducing unwanted sequencing reads of freshly obtained HGSOC. (A) UMAP plot of a CSCK-processed sample with annotated cell types based on transcriptomic data. (B) UMAP plots comparing single-cell RNA sequencing data from unprocessed (Ori) and CSCK-processed samples (CSCK), highlighting hemoglobin (HBB)-positive reads in red that interfere with other populations as seen in the Ori sample. (C) Percentage of mitochondrial (mt) reads in unprocessed (Ori) and CSCK-processed samples, demonstrating a significant reduction in mt reads in CSCK-processed samples (n = 3).

Advantages of the CSCK cleanup solution
Harsh solutions such as RBC lysis solutions are the current standard for removing RBCs from dissociated human tissue samples and can have a significant impact on cell viability compared to magnetic MicroBead-based separation.
To evaluate this, the cryopreserved and CSCK-processed HGSOC samples were compared with samples that underwent a two-step cleanup workflow of dead cell removal and red blood cell lysis. Figure 5 shows both critical cleanup steps prior to downstream sequencing applications.

In single-cell genomics, time matters. The sooner the analysis can be conducted, the fewer transcriptomic changes will be observed. To demonstrate the advantages of using the CSCK one-step cleanup method over a two-step approach, the time required for each step was documented. The CSCK-based one-step protocol took around 40 minutes, whereas the conventional two-step method required about 65 minutes. This demonstrates that the one-step approach reduces processing time by nearly 40%. Even greater time savings are possible if RBC lysis needs to be repeated due to an uncomplete lysis.

Both samples were compared side-by-side to assess the performance of the sample preparation process. Overall viability (fig. 6A), RBC count (fig. 6B), and RNA integrity number (RIN) values (fig. 6C) were measured. The comparison shows that the viability of the CSCK-processed samples was substantially higher (93%) compared to the two-step workflow (Comp, 68%). RBC contamination was reduced from 70% to 13% with the CSCK, meeting the required threshold of not exceeding a frequency of 20%. Interestingly, the samples processed with the two-step workflow failed to reach the 20% RBC frequency threshold recommended by sequencing vendors. The improved sample quality was also validated by the RNA quality, visualized by higher RIN values after CSCK usage compared to the other workflow.

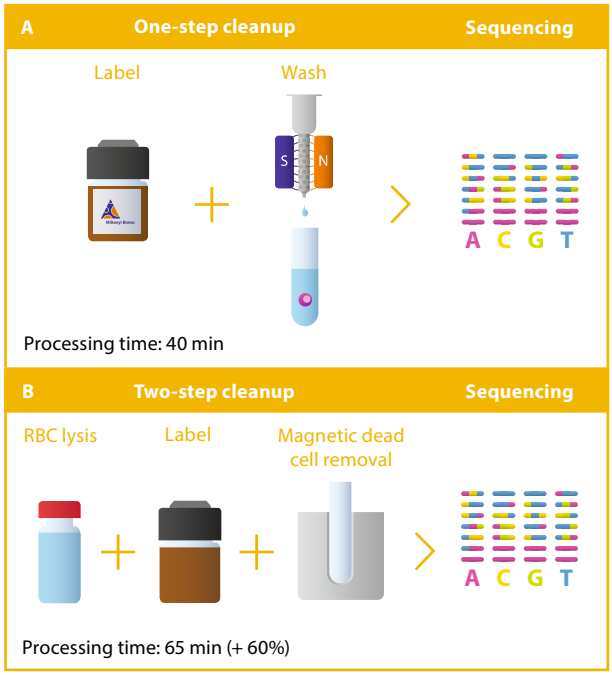

Figure 5: One-step and two-step cleanup workflow comparison: (A) Sample cleanup using the one-step CSCK workflow. (B) Sample cleanup using a commercially available workflow with a two-step protocol for the removal of RBCs and dead cells before downstream analysis. CSCK workflow saves 25 min compared to the two-step workflow.

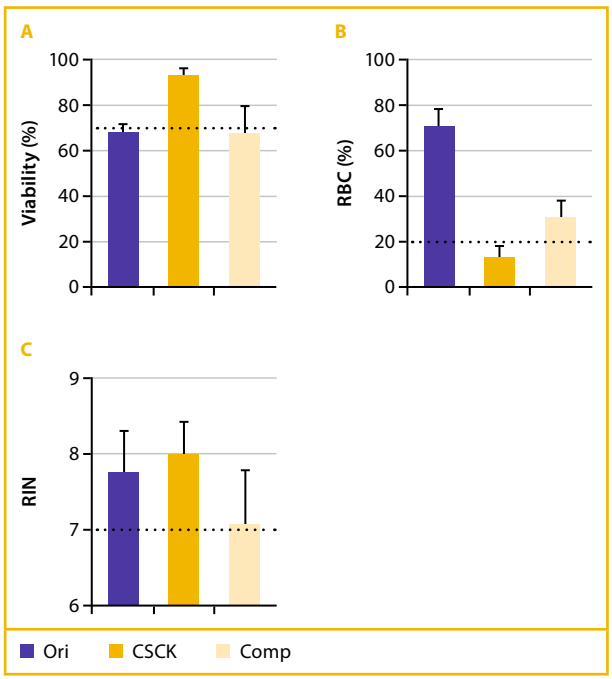

Figure 6: Comparison of basic parameters between no cleanup (Ori), one-step cleanup workflow (CSCK), and two-step cleanup workflow (Comp) of HGSOC samples. (A) Percentage of viable cells after processing different cleanup methods. (B) RBC content after processing different cleanup methods, with a threshold set at 20%, as recommended for single-cell RNA sequencing.[1] (C) Measured RIN values for the original fraction and different processing methods, with a threshold set at 7 based on conventional guidelines (n=3).

RBC depletion prevents unwanted transcriptional changes
Using the one-step instead of the two-step workflow, not only speeds up the entire process of general sample cleanup, but also protects the cells from the unwanted negative effects of aggressive treatment with RBC lysis solutions. Figure 7 shows the number of differentially expressed genes between the two workflows compared to an unprocessed sample.
The unprocessed sample is considered fresh because the RNA was extracted from it directly without additional treatment after tissue dissociation.The number of up- or down-regulated genes in the sample processed by CSCK was significantly lower, at a total of 52 with 36 differentially expressed genes, compared to samples processed using the two-step workflow (Comp), which had a total of 281 and 265 differentially expressed genes. These results underscore the advantages of the one-step CSCK workflow in preserving gene expression profiles closer to the native state, making it a more reliable choice for downstream transcriptomic analyses.

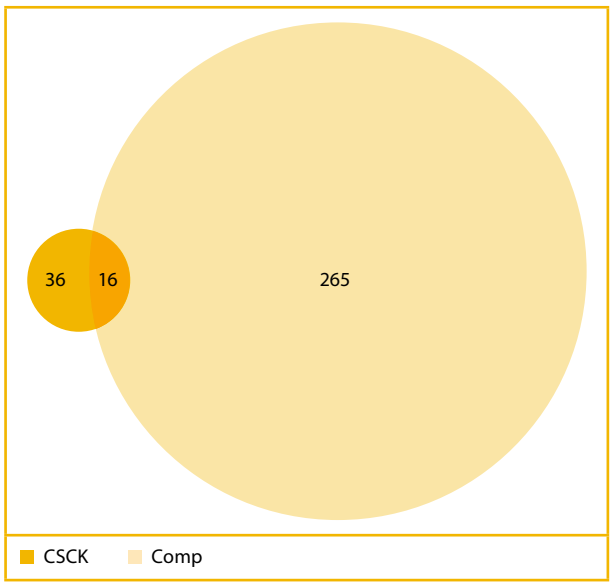

Figure 7: Comparison of gene expression changes between the two workflows. Venn diagram illustrating differentially expressed genes across two processing methods: the one-step workflow (CSCK, dark yellow) or the commercially available two-step workflow (Comp, light yellow) in comparison to a freshly dissociated sample. Overlapping regions (dark orange) represent shared differentially expressed genes, while unique sections highlight method-specific gene expression profiles (n = 3).

Conclusion

The study demonstrates the effectiveness of the CSCK as a one-step MicroBead-based solution for cleaning dissociated human tumor tissue samples prior to scRNA-seq. By reducing unwanted events resulting from, e.g., debris, dead cells, red blood cells, and cell-free organelles, CSCK significantly improves sample quality, resulting in more reliable sequencing data with minimized technical noise while reducing experimental costs. Compared to traditional workflows, the CSCK approach preserves cellular integrity and minimizes stress-induced gene deregulation, leading to more accurate data interpretation and the inclusion of more results, which is particularly important when studying rare cell populations. This workflow provides a streamlined, reliable, and powerful sample preparation option for single-cell genomics applications.

- Unlike the two-step workflow, the one-step cleanup with CSCK does not require the use of harsh RBC lysis, which positively affects transcriptomic stability.
- Reduced mitochondrial reads and RBC interference improve sample quality and sequencing accuracy.
- Reduction of hands-on time saves resources and prevents the cells from changes in gene expression by combining a two-step protocol into a single step.
- The MicroBead-based workflow increases reproducibility and reduces manipulation of gene expression profiles, which is critical for downstream analysis.

Product	Order no.
gentleMACS™ Octo Dissociator with Heaters	130-134-029
Tumor Dissociation Kit, human	130-095-929
gentleMACS C Tubes	130-093-237
Cell Suspension Cleanup Kit, human	130-135-177
MACS Freezing Solution	130-129-552
MACS SmartStrainers (70 µm)	130-098-462
MACSQuant 10 Analyzer	130-096-343
CD45(REA747) – VioGreen, REAfinity	130-110-638
Annexin V-FITC Kit	130-092-052
CD326 (EpCAM) Antibody, PE, anti-human, REAfinity	130-110-999
CD90 Antibody, APC, anti-human, REAfinity	130-114-861
CD31 Antibody, PE-Vio 770, anti-human	130-110-671
CD235a (Gly A) Antibody, APC-Vio 770, anti-human, REAfinity	130-120-471
DAPI Staining Solution	130-111-570
TOM22 Antibody, anti-human/mouse, PE	130-124-240
REAfinity Recombinant Antibodies*	

* For more information on REAfinity Recombinant Antibodies conjugated to a wide range of fluorochromes visit **www.miltenyibiotec.com/antibodies**

References

1. Should I deplete red blood cells from my sample before loading? – 10× Genomics: https://kb.10xgenomics.com/hc/en-us/articles/360002034971 (Accessed May, 2025)

Notes

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