RESEARCH ARTICLE

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Optimal whole blood dilution protocol for preprocessing samples prior to circulating tumor cell capture by size-based isolation

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Abstract

Background: This study compared whole blood dilution versus density gradient centrifugation for pre-processing blood samples prior to circulating tumor cell (CTC) capture on the efficiency of CTC separation by size-based isolation.

Materials and methods: Whole blood from a healthy volunteer spiked with SKBR3 cells was used to optimize the whole blood dilution protocol for sample volume, dilution ratio, and paraformaldehyde (PFA) concentration. Whole blood from healthy volunteers spiked with SKBR3, A549, or PC3 cells, and whole blood from patients with advanced gastric, esophageal, or liver cancer, was used to compare pre-processing by the optimal whole blood dilution protocol with density-gradient centrifugation. All statistical evaluations were performed using Student t test of the Statistical Package for Social Sciences (SPSS version 17.0).

Results: In blood samples from healthy volunteers, spiked SKBR3 cell recovery rates were highest in 5 ml of whole blood, diluted with 2.5 ml buffer, and fixed with 0.2% PFA, and spiked SKBR3, A549, and PC3 cell recovery rates from 5 ml whole blood were significantly greater when using the optimized whole blood dilution protocol (87.67% ±1.76%, 79.50% ±0.50% and 71.83% ±1.04%, respectively) compared to density-gradient centrifugation (46.83% ±1.76%, 37.00% ±1.50% and 41.00% ±1.50%, respectively).

KEYWORDS
blood dilution, circulating tumor cell, circulating tumor microemboli, size-based isolation

1 | INTRODUCTION

Circulating tumor cells (CTCs) are tumor cells that enter the peripheral blood. The existence of CTCs or circulating tumor microemboli (CTM) is considered a biomarker of tumor recurrence and metastasis,[1–3] and large number of CTCs are associated with shorter overall and progression free survival.[4] In recent years, a large number of clinical studies have indicated that CTCs play an important role in prognosis, response to therapy, tumor staging, and prediction of recurrence and metastasis.[5]

The vast majority of disseminating tumor cells may be cleared by anoikis/apoptosis, shear stress, or the immune system, and only a fraction of CTCs can survive. As CTCs are rare, their separation and enumeration are challenging. Approaches to CTC separation/enrichment are based on CTCs biological or physical properties. Specifically, the CellSearch™ System utilizes CTCs biological

properties. The CellSearch™ System targets the EpCAM antigen for capturing CTCs and received FDA approval for clinical diagnosis of metastatic breast cancer, colorectal cancer, and prostate cancer in 2004 and 2008.[6–9] Separation/enrichment methods based on physical properties use size-based isolation. The approach is label free, which relies on filtration and differences in size and deformity between CTCs and blood cells, and compatible with detailed molecular and functional assessments.[10] Size-based isolation methods had been successfully applied to the study of CTCs in patients with melanoma, breast, lung, and hepatocellular carcinomas.[10–14]

Procedures for processing the blood samples prior to CTC separation by size-based isolation vary. Some rely on dilution while others require density-gradient centrifugation. The objective of this study was to compare whole blood dilution versus density-gradient centrifugation for pre-processing blood samples prior to CTC capture on the efficiency of CTC separation by size-based isolation.

2 | MATERIALS AND METHODS

2.1 | Cell lines and patients

SKBR3 (human breast cancer cell line), A549 (human lung cancer cell line), and PC3 (human prostate cancer cell line) cells were routinely cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 5% fetal bovine serum (Sigma-Aldrich). Following culture, cells were harvested using 0.05% trypsin (Sigma-Aldrich). Cell suspensions were used when their viability exceeded 95% as assessed by trypan blue exclusion.

Thirty patients with advanced gastric cancer, esophageal cancer, or liver cancer who were being treated at Shandong Cancer Hospital affiliated to Shandong University were recruited between March and May 2017. Included patients must be in advanced stage cancer and have only one type of primary malignant tumor diagnosed. Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of Shandong Cancer Hospital affiliated to Shandong University.

2.2 | Study design

The flow chart of the study was shown in Figure 1. The whole blood dilution protocol was optimized using 120ml of peripheral blood that was collected from a single healthy volunteer into regular ethylene diamine tetraacetic acid collection tubes (EDTA tube, BD Vacutainer® REF: 367863) and pooled. Whole blood dilution (WBD) buffer was prepared by adding EDTA and bovine serum albumin (BSA) to pH 7.4 phosphate buffer saline (PBS) buffer (final concentration 1mM EDTA and 1% BSA). Optimal blood volume, dilution ratio, and paraformaldehyde (PFA) concentration were explored using 1-,
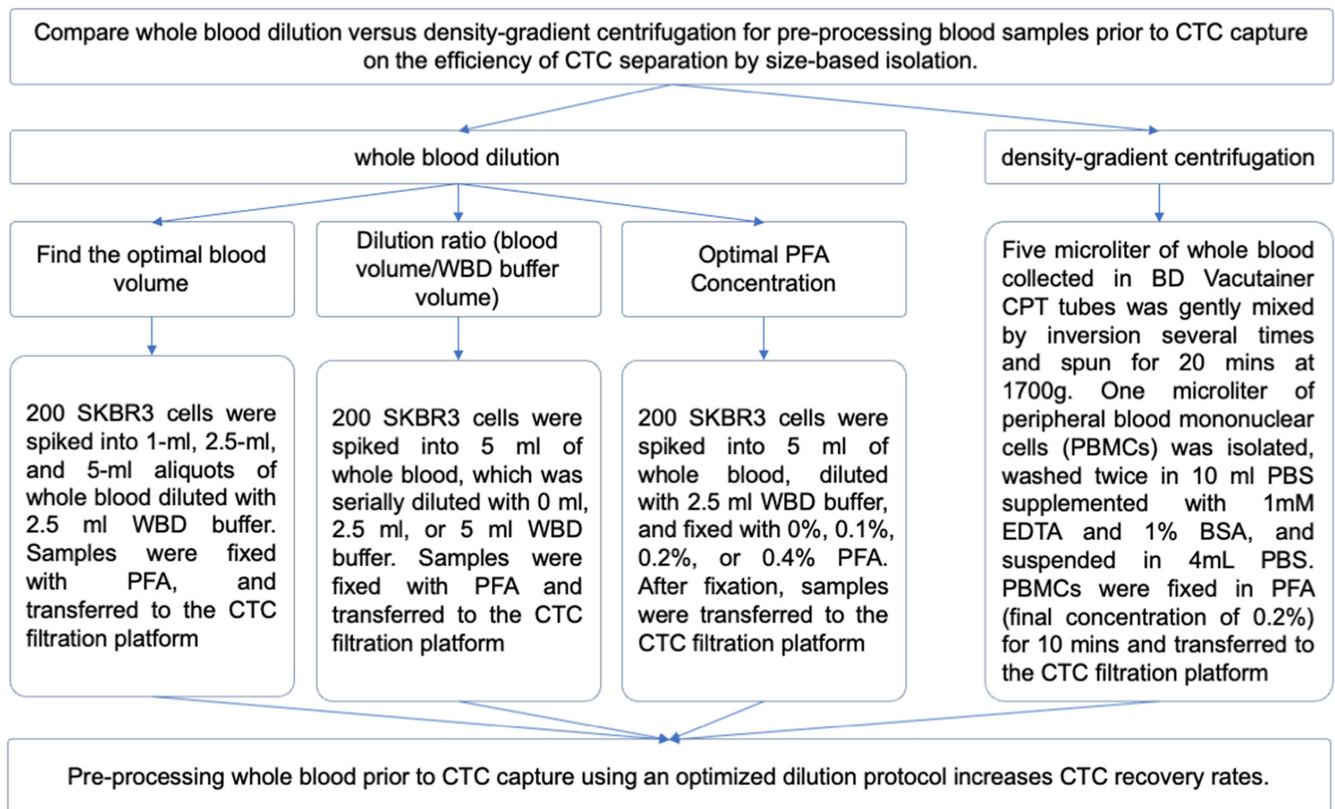

FIGURE 1 Overall flow chart of the study design

2.5-, and 5-ml aliquots of whole blood spiked with 200 SKBR3 cells. Measurements were performed in triplicate.

The optimized whole blood dilution protocol versus density-gradient centrifugation for pre-processing blood samples was compared using 90 ml of peripheral blood collected from healthy volunteers that was pooled, divided into 5-ml aliquots, and spiked with 200 SKBR3 cells, 200 A549 cells, or 200 PC3 cells. Detection sensitivity was assessed using 10, 50, 200, or 400 SKBR3 cells spiked into 5 ml of whole blood. Measurements were performed in triplicate.

The optimized whole blood dilution protocol versus density-gradient centrifugation for pre-processing blood samples was compared using 12 ml of peripheral blood collected from patients with advanced gastric cancer, lung cancer, or breast cancer before they started treatment. The first 2 ml of blood was discarded to prevent epithelial contamination. Subsequently, 10 ml of blood was equally divided into two portions and each portion was placed in an EDTA tube. One portion was diluted under optimal conditions and the other was subjected to density-gradient centrifugation.

The number of SKBR3 cells, A549 cells, or PC3 cells was determined using the MoFlo XDP Cell Sorter (Beckman Coulter). All samples were processed and analyzed according to the CTC enrichment and enumeration protocol, whereby CTCs were separated by size-based isolation. All procedures were performed by the same operator.

2.3 | Dilution of whole blood

To optimize blood volume, 200 SKBR3 cells were spiked into 1-, 2.5-, and 5-ml aliquots of whole blood diluted with 2.5 ml WBD buffer. Samples were fixed with PFA, and transferred to the CTC filtration platform. To optimize the dilution ratio, 200 SKBR3 cells were spiked into 5 ml of whole blood, which was serially diluted with 0, 2.5, or 5 ml WBD buffer. Samples were fixed with PFA and transferred to the CTC filtration platform. To optimize PFA concentration, 200 SKBR3 cells were spiked into 5 ml of whole blood, diluted with 2.5 ml WBD buffer, and fixed with 0%, 0.1%, 0.2%, or 0.4% PFA. After fixation, samples were transferred to the CTC filtration platform.

2.4 | Density-gradient centrifugation

Five microliter of whole blood collected in BD Vacutainer CPT tubes was gently mixed by inversion several times and spun for 20 min at 1700 g. One microliter of peripheral blood mononuclear cells (PBMCs) was isolated, washed twice in 10 ml PBS supplemented with 1 mM EDTA and 1% BSA, and suspended in 4 ml PBS. PBMCs were fixed in PFA (final concentration of 0.2%) for 10 min and transferred to the CTC filtration platform.

2.5 | CTC enrichment and enumeration using size-based isolation

The method for size-based isolation of CTCs was adapted from previously published techniques.[11,15] Briefly, a 2.5 or 5 ml whole blood sample was either pre-processed by the optimal whole blood dilution protocol or density-gradient centrifugation. Fixed cells were transferred to the CTC filtration platform, which included a polycarbonate membrane (pore size 8 μm). Captured cells were isolated, enriched, and stained with May-Grunwald-Giemsa. The membrane was examined microscopically. CTCs were characterized by the presence of at least 4 of the following criteria[15]: irregular nuclei, prominent nucleoli, nuclear diameter > 18 μm, nuclear-cytoplasmic ratio > 0.8, hyperchromatic nuclei, nonhomogeneous staining, irregular nuclear membrane, or CTM. All suspected CTCs/CTM were reviewed and identified independently by 3 cytopathologists blinded to the clinical and pathological status of the blood samples tested.

2.6 | Statistical methods

All statistical evaluations were performed using the Statistical Package for Social Sciences (SPSS version 17.0). Student t test was used to evaluate differences in percent cell recovery. Statistical significance was reached at $p < 0.05$.

3 | RESULTS

3.1 | Optimizing whole blood dilution

The whole blood dilution protocol was optimized using blood collected from a single healthy volunteer that was divided into aliquots and spiked with 200 SKBR3 cells.

When optimizing blood volume, the spiked SKBR3 cell recovery rates were 69.83 ± 0.76%, 75.00 ± 2.29%, and 79.67 ± 2.02%, for 1, 2.5, and 5 ml of whole blood, respectively (Figure 2A). Recovery rates at 5 and 2.5 ml of whole blood were significantly greater than at 1 ml of whole blood.

When optimizing the dilution ratio, the spiked SKBR3 cell recovery rates were 78.00 ± 4.36%, 79.67 ± 4.80%, and 68.00 ± 5.57% for dilution ratios (blood volume/WBD buffer volume) 5 ml/0 ml, 5 ml/2.5 ml, and 5 ml/5 ml, respectively (Figure 2B). There were no significant differences between the recovery rates, but as the dilution ratio increased, the number of white blood cells captured by the filter was significantly reduced.

When optimizing the PFA concentration, the spiked SKBR3 cell recovery rates were 33.00 ± 0.50%, 48.00 ± 5.50%, and 79.67 ± 1.76%, at 0%, 0.1%, and 0.2% PFA, respectively (Figure 2C). Recovery rates were significantly improved when PFA concentration was increased from 0% to 0.2%, but when PFA concentration reached 0.4%, the fixed diluted whole blood clogged the filter.
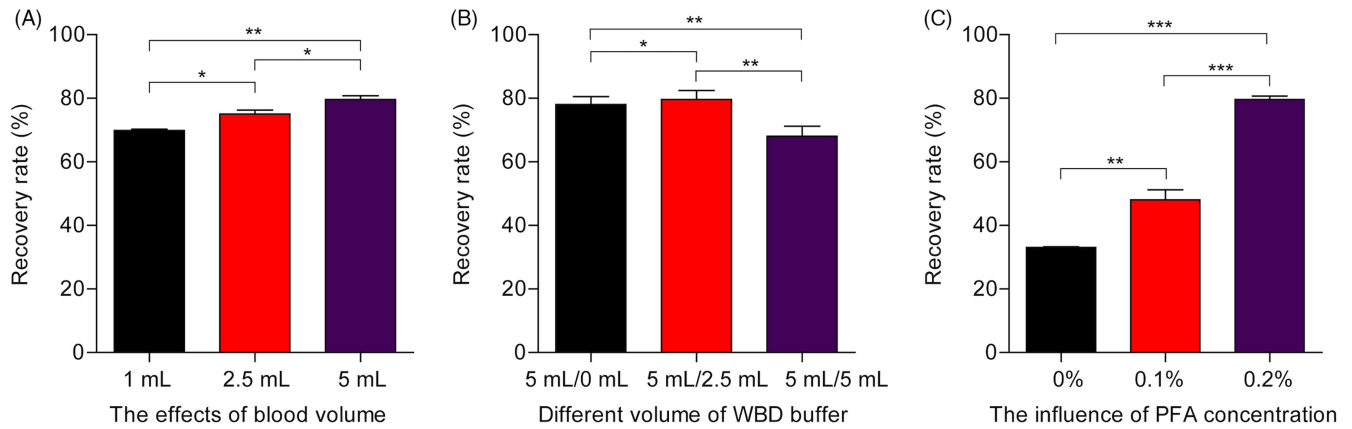
FIGURE 2 Optimization of the whole blood dilution protocol: (A) The spiked SKBR3 cell recovery rate stratified by sample volume; (B) the spiked SKBR3 cell recovery rate stratified by dilution ratio; (C) the spiked SKBR3 cell recovery rate stratified by PFA concentration
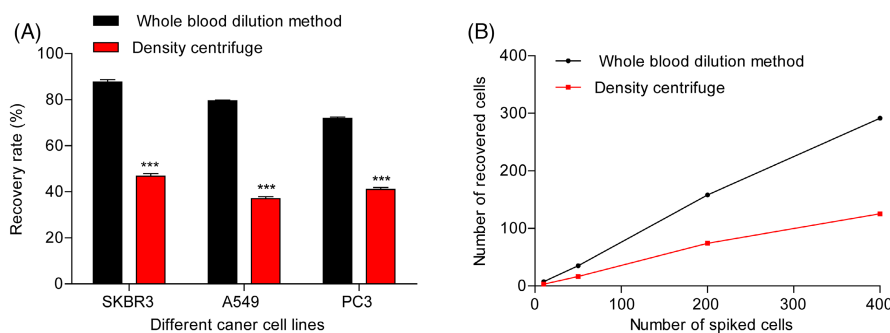
FIGURE 3 Comparison of whole blood dilution versus density-gradient centrifugation for pre-processing blood samples prior to CTC capture on the efficiency of CTC separation by size-based isolation: (A) Spiked SKBR3, A549, or PC3 cell recovery rates; (B) sensitivity of whole blood dilution versus density-gradient centrifugation using SKBR3cells

Spiked SKBR3 cell recovery rates were highest in 5 ml of whole blood, diluted with 2.5 ml WBD buffer, and fixed with 0.2% PFA. These conditions were used in subsequent experiments.

3.2 | Comparison of pre-processing methods

A comparison of the optimized whole blood dilution protocol with density-gradient centrifugation for pre-processing blood samples prior to CTC capture was performed. Findings showed spiked SKBR3, A549, and PC3 cell recovery rates from 5 ml of whole blood were significantly greater when using the optimized whole blood dilution protocol ($87.67\% \pm 1.76\%$, $79.50\% \pm 0.50\%$ and $71.83\% \pm 1.04\%$, respectively) compared to density-gradient centrifugation ($46.83 \pm 1.76\%$, $37.00 \pm 1.50\%$ and $41.00 \pm 1.50\%$, respectively) (Figure 3A).

Detection sensitivity of the optimized whole blood dilution protocol was significantly higher than for density-gradient centrifugation. Among the 5 ml of whole blood spiked with 0, 50, 200, or 400 SKBR3 cells, the optimized whole blood dilution protocol detected 7.30 ± 0.58, 35.00 ± 1.00, 158.67 ± 1.53, and 291.33 ± 5.86 SKBR3 cells, with spiked SKBR3 cell recovery rates of $73.33 \pm 5.77\%$, $70.00 \pm 2.00\%$, $79.00 \pm 1.00\%$, and $72.83 \pm 1.46\%$, respectively. Density-gradient centrifugation detected 3.33 ± 0.58, 16.33 ± 1.53, 74.00 ± 4.00, and 125.67 ± 3.79 SKBR3 cells, with spiked SKBR3 cell

recovery rates of $33.33 \pm 5.77\%$, $32.67 \pm 3.06\%$, $37.00 \pm 2.00\%$, and $31.42 \pm 0.95\%$, respectively (Figure 3B).

3.3 | Detection of CTCs in patients

CTCs were detected in blood samples collected from patients with various metastatic cancers. Of 30 blood samples pre-processed using the optimized whole blood dilution protocol, 22 patients had detectable CTCs, and 5 patients had CTM (range 0–3). Mean CTCs/5 ml whole blood was 2.30, median CTCs/5 ml whole blood was 2, and the range was 0–12. Of 30 blood samples pre-processed using density-gradient centrifugation, 5 patients had detectable CTCs (CTC ≥1) and 1 patient had 4 CTM. Mean CTCs/5 ml whole blood was 0.50, median CTCs/5 ml whole blood was 0, and the range was 0–5 (Figure 4).

4 | DISCUSSION

CTCs are malignant cells shed from primary tumors or metastases into the peripheral blood circulation. CTCs give rise to distant metastases that are usually the cause of cancer-related mortality. In recent years, liquid biopsy has been used to identify CTCs in many clinical applications. However, CTCs are rare, with concentrations as

FIGURE 4 CTC detection in 30 patients with advanced cancer after pre-processing blood samples by the optimized whole blood dilution protocol or density-gradient centrifugation

low as 1 CTC/10^5–10^7 mononuclear cells in the peripheral blood of cancer patients; therefore, it is necessary to develop effective and consistent methods for CTC separation and enrichment.

This study used an automated size-based isolation system to separate and stain CTCs. The method utilizes 8 μm pore filters to separate larger CTCs from smaller leukocytes, and was adapted from previously published reports.[7–11] The current study compared different methods for pre-processing blood samples prior to CTC capture on the efficiency of CTC separation by this size-based isolation system. Traditionally, blood has been pre-processed by red blood cell (RBC) lysis[11] or density-gradient centrifugation. We did not investigate RBC lysis because in our size-based isolation system, it has a negative effect on downstream staining of CTCs following enrichment (data not shown). As an alternative to RBC lysis, density-gradient centrifugation with Ficoll–Hypaque can be used to separate CTCs and PBMCs from blood cells and granulocytes according to their density. However, findings from this study showed that spiked cancer cell line recovery rates and the number of CTCs/CTM identified in blood samples collected from patients with advanced cancer were low when density-gradient centrifugation was used for pre-processing blood samples prior to CTC capture.

During density-gradient centrifugation, CTCs may be lost due to the migration of cells along the density gradient or the presence of aggregates. Therefore, we evaluated whole blood dilution as an alternative pre-processing technique. Whole blood is 60% plasma and 40% cells. The cellular component consists of erythrocytes (~99%), white blood cells, platelets, and rare-cells such as CTCs.

When using size-based isolation to separate/enrich CTCs in whole blood, issues arise from clogging while filtering, and purity can be compromised by an abundance of white cells. Findings from the present study suggest that clogging can be eliminated and purity enhanced by diluting the blood sample prior to CTC capture, and an optimal protocol for diluting blood samples was developed. The WBD buffer consisted of PBS supplemented with 1 mM EDTA

and 1% BSA to prevent agglomeration and adsorption to micro-channel walls and maintain captured cells intact.[16] The blood volume processed did not influence the spiked SKBR3 cell recovery rate. When optimizing the dilution ratio, the number of white blood cells on the filter was obviously increased when whole blood was not diluted, but the spiked SKBR3 cell recovery rate did not vary with the dilution ratio. The concentration of PFA was found to be crucial. Inadequate fixation allowed CTC cells to pass through the 8 μm pores because viable cells can change their shape when subjected to extra pressure. This led to dramatically decreased spiked cancer cell line recovery rates. Conversely, at 1% PFA, white blood cells occupied the pores and eventually clogged the filter. Finally, 0.2% PFA was considered optimal. Use of the optimized whole blood dilution protocol to pre-process blood samples from patients with advanced cancer prior to CTC capture significantly increased the number of CTCs/CTM detected compared to density-gradient centrifugation.

In conclusion, this study demonstrated that pre-processing whole blood prior to CTC capture using an optimized dilution protocol increases CTC recovery rates. Dilution of samples is a convenient way to process blood before CTC separation/enrichment by automated size-based isolation.

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