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The RareCyte® Platform for Next-Generation Analysis of Circulating Tumor Cells

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Abstract

Circulating tumor cells (CTCs) can reliably be identified in cancer patients and are associated with clinical outcome. Next-generation "liquid biopsy" technologies will expand CTC diagnostic investigation to include phenotypic characterization and single-cell molecular analysis. We describe here a rare cell analysis platform designed to comprehensively collect and identify CTCs, enable multi-parameter assessment of individual CTCs, and retrieve single cells for molecular analysis. The platform has the following four integrated components: 1) density-based separation of the CTC-containing blood fraction and sample deposition onto microscope slides; 2) automated multiparameter fluorescence staining; 3) image scanning, analysis, and review; and 4) mechanical CTC retrieval. The open platform utilizes six fluorescence channels, of which four channels are used to identify CTC and two channels are available for investigational biomarkers; a prototype assay that allows three investigational biomarker channels has been developed. Single-cell retrieval from fixed slides is compatible with whole genome amplification methods for genomic analysis. © 2018 The Authors. Cytometry Part A published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

Key terms

liquid biopsy; circulating tumor cells; cell separation; immunofluorescence microscopy; molecular imaging; image processing; computer assisted; single-cell analysis; DNA amplification techniques.

"Liquid Biopsy"—Requiring More from Less

Over the past century, the medical approach to accessing tumors for analysis has evolved from highly invasive to increasingly less invasive: from surgical resection to excisional biopsy, to core needle biopsy, and to fine needle aspirate. Yet as biopsy sample volume has decreased, the diagnostic information required from the sample has increased. The least invasive access to tumor cells is by "liquid biopsy," a sampling method to collect rare circulating tumor cells (CTCs) in the blood (1,2). The promise of liquid biopsy is that real-time blood sampling can provide the depth of data necessary for 21st century clinical investigation and patient care. The current challenge is to extract the required biological information from these few rare cells.

The CellSearch® system established that CTCs can be reliably identified in the blood of cancer patients and that the number of CTCs is strongly associated with clinical outcome (3–10). Despite its groundbreaking technology, CellSearch has historically been limited by the immuno-magnetic method it employs to collect and identify EpCAM-expressing CTCs and by the lack of an integrated single-cell isolation capability.

Traditional tissue biopsy samples are processed using formalin fixation and paraffin embedding. Sections are cut from the paraffin block and placed onto a microscope slide for analysis. The tissue slide provides information at the following three

levels: 1) *identification* of cancer; 2) *phenotypic characterization* of cancer; and 3) *molecular analysis* of cancer. The future of cell-based liquid biopsy will involve expanding the breadth and depth of CTC-derived information from the first level to the second and third levels. The rare cell analysis platform presented here provides such information.

Working Principles

The RareCyte platform comprises buffy coat expansion and spreading onto slides, automated multiparameter fluorescence staining, automated microscopic imaging and analysis, and integrated single-cell retrieval. The working principle that enables the comprehensive collection of CTCs is the differential density of the nucleated cell buffy coat layer from red blood cells (RBCs) and plasma. The method is based on buffy coat "expansion," introduced by Levine and Wardlaw as the quantitative buffy coat (QBC) system (11). In the QBC, a float with defined density is placed within a capillary tube, which is filled with a blood sample. After centrifugation of the tube, the buffy coat is "expanded" in the space surrounding the float; the number of platelets and leukocytes can be calculated by determining the linear space occupied after imaging.

The same "expansion" concept can be scaled for larger blood volumes. The platform uses this principle to separate nucleated cells by density and, then, transfer them to microscope slides. The four components of the system are described below; associated methods have been published in detail (12–15).

Sample Preparation System

The sample preparation system enables comprehensive and reproducible collection and transfer of nucleated cells from blood to microscope slides (Fig. 1A–D). Since CTCs are nucleated cells, their density falls within the range of white blood cells (WBCs), which are denser than plasma, but less dense than RBCs. The system collects cells by density, without regard to size or protein expression. This overcomes limitations of filters and microfluidic devices that will fail to collect CTCs that are not larger than WBCs, and surface immunocapture methods that rely on proteins that may not be expressed on all CTCs.

Whole blood is collected in the RareCyte blood collection tube (BCT) and is stable for up to 72 h (Fig. 1A). Blood is placed in a separation tube containing an internal float having density similar to nucleated blood cells. After centrifugation, the buffy coat is "expanded" within the space between the tube and the float, above the RBC layer, and below the plasma. An external sealing ring is applied that clamps the tube against the float to create a physical barrier between the RBC and buffy coat layers. The plasma is collected and may be used for cell-free DNA analysis. A displacement fluid (~1.13 g/cm^3) is added above the float, and a collector is inserted that connects the contents of the separation tube to an isolation tube containing a slightly less dense fluid (~1.10 g/cm^3) that further separates the blood components. The collector allows movement of fluid and cells between the separation tube and the isolation tube. During a second centrifugation, the desired nucleated cell layer, which is less dense than the isolation fluid, is buoyantly displaced into the isolation tube, to collect the nucleated cells in a small volume (~120 μl). A transfer fluid containing a nonformalin fixative is added, and the resulting mixture is spread onto eight microscope slides using a device that draws the fluid into a monolayer smear by surface tension between its spreading blade and the slide (Fig. 1B).

The system is designed to collect platelets along with nucleated cells while removing 99.5–100% of RBCs. The absence of RBC lysis or wash steps minimizes cell loss. The processing time from blood to slide is about 75 min. Prepared slides are air dried for 30 minutes and can be stored frozen for 1 year or longer before staining. Components of the system are shown in Supporting Information Figure S1.

Automated Fluorescence Staining of Microscope Slides

The prepared slide contains CTCs, if present, in a background of WBCs. Once on the slide, CTCs may be identified, characterized by phenotype, and analyzed at a molecular level. The RareCyte platform workflow uses automated staining instruments (made by Ventana, Dako, and Leica) to stain slides by immunofluorescence. This increases laboratory throughput, eliminates variability, and increases control of the staining conditions.

The four canonical markers used for the identification of epithelial CTCs are a nuclear dye, the WBC exclusion marker CD45, and the epithelial CTC markers EpCAM and cytokeratin (CK). Since the imaging system can analyze six fluorescence channels, two or three additional investigational biomarkers may be simultaneously assessed on the identified CTCs, depending on whether the epithelial markers are in separate channels or combined into one (see Fig. 3).

Imaging System

Stained slides are placed into the platform's automated fluorescence scanning microscope and scanned at 10× objective magnification. The microscope stage employs a kinematic mount that establishes highly reproducible positioning of the slide; X–Y displacement upon reloading is approximately 2–3 μm. Scanning of each slide in four channels takes about 12 min inclusive of image plane determination (Supporting Information Fig. S3); scanning all eight slides from a 7.5-ml sample thus takes ~1.5 h. To increase imaging throughput, an automated slide loader for up to 80 slides is being developed (Supporting Information Fig. S2).

Slide images are analyzed with integrated image analysis software (Fig. 1D and Supporting Information Fig. S2) that automatically analyzes the images to find and score CTC candidates in about 10 min as follows. Objects with signal in CK or EpCAM are identified. These are then screened by the software for CD45 signal, which, if present, causes the object to be rejected. This excludes virtually all WBCs and results in a set of CTC candidate objects. Candidate objects are ranked by a machine-learning model based on a large set of image

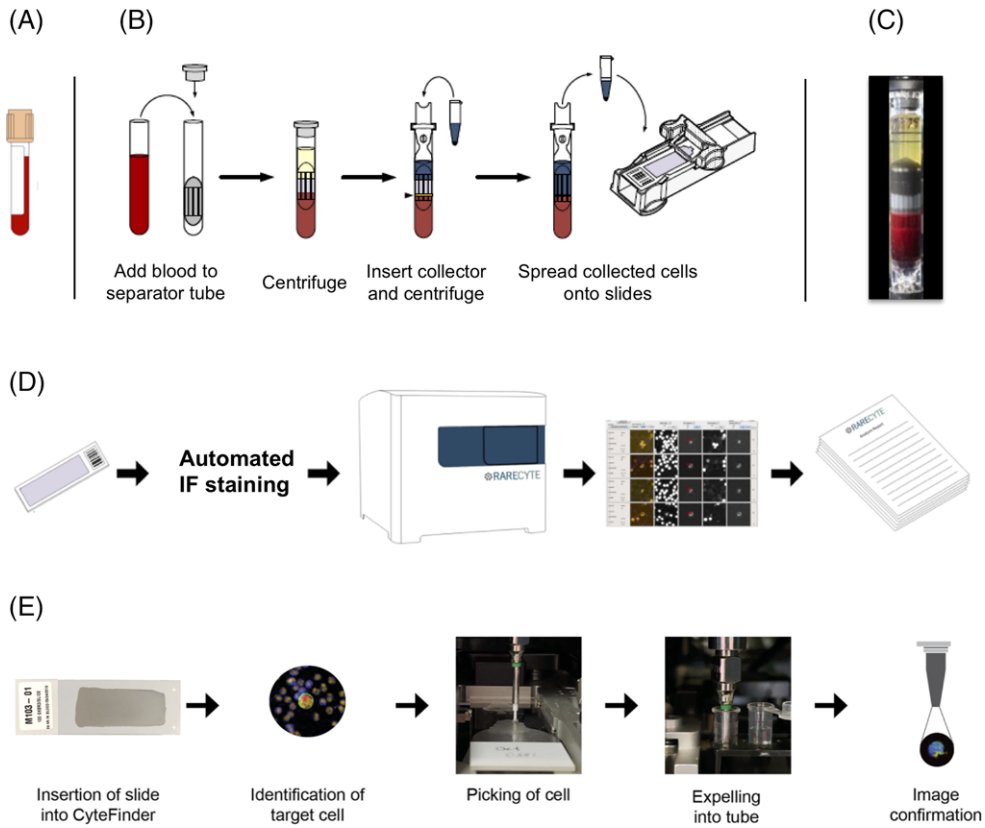

Figure 1. RareCyte platform CTC sample preparation, staining, and analysis workflow. (**A**) Blood is collected into the AccuCyte BCT that preserves samples for processing up to 72 h. (**B**) Diagram of steps in density-based collection of nucleated cells and transfer to microscope slide. (**C**) Photograph of AccuCyte separation tube after initial centrifugation, demonstrating fractionation of blood into plasma (top, yellow), red blood cell layer (bottom, red), and nucleated cell layer containing CTCs (gray-white band around black float). (**D**) Diagram of subsequent CTC analysis workflow: automated staining of slides, automated scanning image acquisition and machine learning image analysis by the CyteFinder system, candidate CTC review and confirmation, and report generation. (Note: retrieval of individual CTCs by the CytePicker module is not shown.)

features that determines a probability score (0–100) for being a "true" CTC. The model is trained on a data set of >10,000 CTCs from various cancer types that have been observer confirmed to have a "true" epithelial CTC phenotype. Candidate object images are presented to the reviewer in order of their score, from high to low, ending at a threshold cutoff score of 15 that was selected to retain approximately 99% of "true" CTCs while excluding approximately 90% of "false" candidate objects in the data set. Probability score ranking increases the concordance between reviewers and decreases review time: low-scoring objects may be screened quickly, since CTCs are rarely present at end of the list. A typical slide generates ~300 candidate cell objects that are displayed for reviewer confirmation; per-slide review time is 3–5 min.

Final classification of a cell as a CTC requires reviewer confirmation of nuclear fluorescence signal, signal for either CK or EpCAM or both, and absence of CD45 signal. Single-cell molecular studies have identified mutations that demonstrate the malignant origin of CTCs so classified (see below). Nuclear morphology may be used to discriminate between CTCs and WBCs if dim CD45 signal is observed on a cell with epithelial marker staining.

Single Cell Retrieval System

The imaging instrument incorporates a fluid-coupled picking system above the slide stage for retrieval of individual CTCs (Fig. 1E). Its needle tip mechanically dislodges the cell from the slide surface and deposits it into an imaging tube for visual confirmation. The system is highly automated and is designed so that it does not require high technical skill; the rate of successful cell retrieval is 80–90%. Because slides may be stored and archived after staining, single CTC can be retrieved for molecular analysis retrospectively. Retrieved cells are suitable for whole genome amplification by methods that are compatible with fixed samples. (A method for preparation and single-cell analysis of live cells using chamber slides has been developed but is not discussed here.) Prepared DNA may be used for various types of analysis including PCR, mass spectrometry, comparative genomic hybridization, and next-generation sequencing (12,15,17). Specific gene sequencing of identified CTCs has confirmed mutations present in tumor tissue samples. Whole exome sequencing has been used to investigate genomic evolution of CTCs during the course of therapy (Supporting Information Figs. S5 and S6) (18).

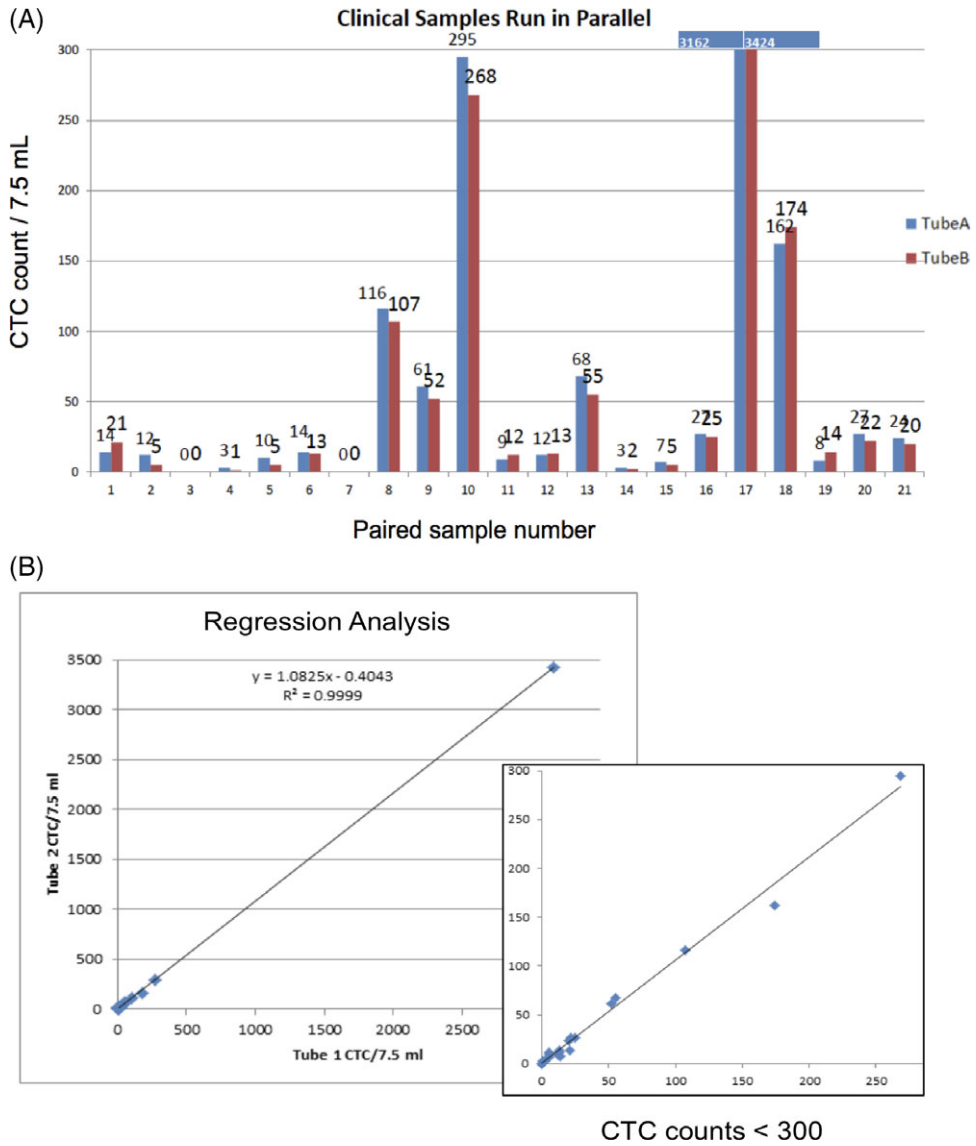

Figure 2. Concordance of CTC counts in paired blood samples. Paired blood samples were collected from 21 patients with advanced cancers (prostate, breast, lung, and Merkel cell). The samples were processed and analyzed using the RareCyte platform laboratory by operators blinded to the sample identity and CTC count result. Samples were randomly assigned as "Tube A" and "Tube B." (**A**) Individual paired sample data demonstrate minimal variability between paired samples. (**B**) Linear regression analysis yielded extremely high correlation between the Tube A and Tube B samples sets. Random permutation resampling analysis yielded additional evidence that test results are highly correlated across the sample set (Supporting Information Fig. S4).

System Performance

Blinded studies using precisely counted spike-in samples of high- and low-EpCAM expressing cell lines yielded highly linear (R^2 = 0.98) recovery of spiked-in cells with mean greater than 90%. Single-digit spike-in recovery was greater than 80%, and solitary spiked-in cells could be detected (12). In blinded comparison against CellSearch, equivalent recoveries were observed in lines that express high EpCAM high levels (MCF7 and LNCaP). However, in low/variable EpCAM-expressing cell lines (PC3 and A549) RareCyte counts were approximately two- to threefold higher than CellSearch counts.

In similar blinded comparison testing of paired breast (n = 24), prostate (n = 17), and lung (n = 12) cancer samples, 55% had equivalent counts with both platforms; 28% had higher RareCyte counts than CellSearch counts by more than 50% (mean approximately twofold, maximum approximately threefold); and 17% had CellSearch counts less than 5 and RareCyte counts 5 or more (19). There was strong correlation between the RareCyte and CellSearch counts in breast (R^2 = 0.94) and prostate (R^2 = 0.83) cancers. However, there was no correlation in lung cancer (R^2 = 0.03), where samples often had no CTCs found by CellSearch; this is consistent with recognized absence of EpCAM in many lung cancers.

When the epithelial phenotype of confirmed clinical CTCs was investigated, the majority of CTCs identified with the RareCyte platform were both CK positive and EpCAM positive, ~25% of cells were CK positive and EpCAM negative, and ~20% of cells were CK negative and EpCAM positive (unpublished). In a triple-negative breast cancer case study, intensive longitudinal monitoring of CTC count was shown to be a sensitive and dynamic correlate of therapeutic response to anticancer therapies, supporting the definition of CTCs employed with the platform (Supporting Information Fig. S5) (20).

Concordance between CTC counts in paired patient samples was assessed in a blinded study (Fig. 2). Linear regression demonstrated very high correlation between sample pairs (R^2 = 0.99). The 1000× random permutation resampling of paired sample order yielded a set of tightly distributed slopes (mean 0.98) and intercepts (mean 0.11) supporting the conclusion that test results for the same patient are highly correlated across the sample set (Supporting Information Fig. S4).

False-positive CTCs are rarely observed. The platform infrequently finds a single cell that fits the classification of a CTC in blood from healthy volunteers. Assessment of CTCs in persons with noncancer diseases has not been performed. Clinical outcome studies to demonstrate the prognostic significance of RareCyte CTC counts are planned but have not been completed. However, sequencing studies of CTCs have provided molecular evidence that cells identified as CTCs by the RareCyte platform are in fact malignant. For example, in a sample from a patient with prostate cancer with known mutation in the *TP53* gene, EpCAM-positive and CK-negative CTCs were retrieved for gene-specific PCR and Sanger sequencing; in five of the eight cells, the mutation was identified (unpublished).

Multi-Parameter Phenotypic Characterization CTCs

The diagnostic value of multiparameter investigation of CTCs is enhanced by the noninvasive nature of blood sampling, which allows access to tumors in patients at risk for procedural complications of biopsy and enables monitoring for dynamic assessment of cancer phenotype. Phenotypic panels can include markers that may be rationally used to inform treatment approach. Such "actionable" information could be provided by assays for proliferation, mesenchymal transition, resistance, and lineage differentiation.

In breast cancer, the primary approaches to therapy remain directed at the estrogen and Her2 pathways (16). Figure 2 shows images generated using a novel six-parameter CTC assay that assesses the expression of estrogen receptor (ER) and Her2 as well as the proliferation marker Ki-67 on breast cancer cell line model CTCs. We have also demonstrated the assessment of potentially actionable markers on prostate CTCs, including androgen receptor (AR) and its splice variant ARv7, and prostate-specific membrane antigen (PSMA). A multiparameter assay developed for the investigation of immune checkpoint inhibitor therapeutic response includes both the well-established marker PD-L1 and the

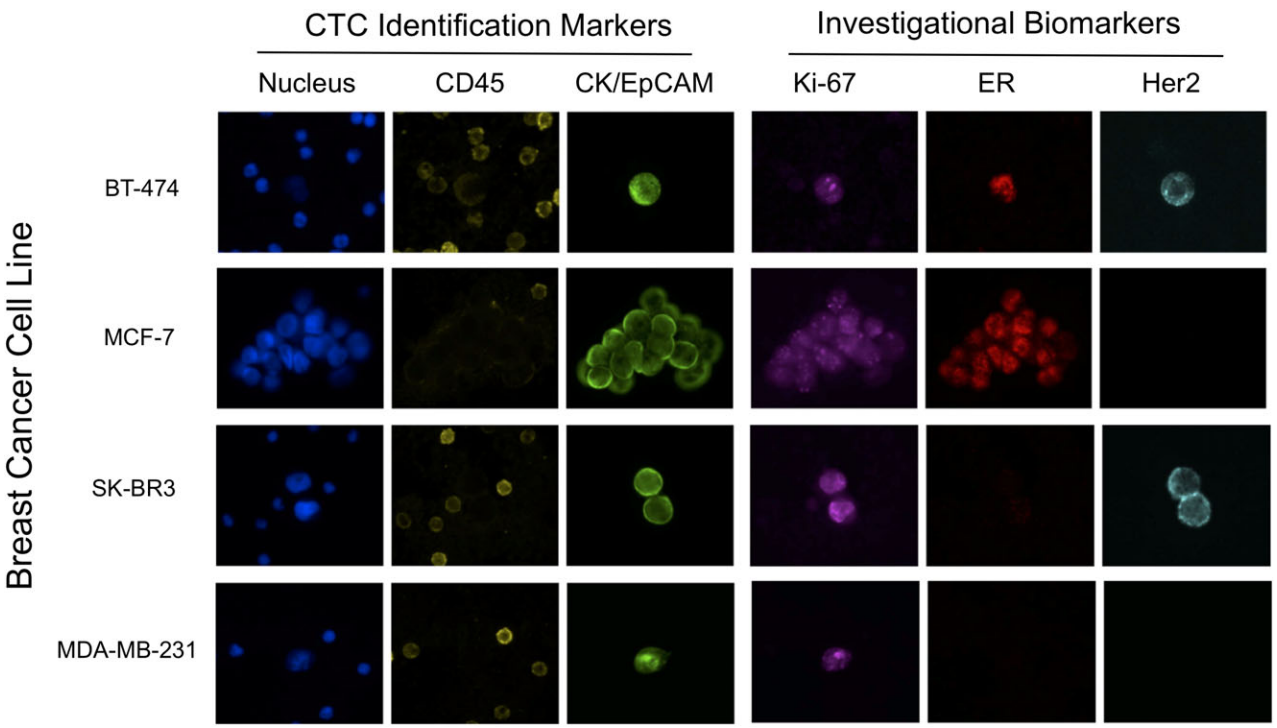

Figure 3. Six-parameter breast cancer CTC assay incorporating investigational biomarkers ER, Her2, and Ki-67. Breast cancer cell lines representing various ER and Her2 phenotypes were spiked into normal donor blood and processed to slides using the AccuCyte system. Model CTCs were identified by positive staining for epithelial markers against CK and/or EpCAM and negative staining for CD45. The ER/Her2 staining pattern of the cell lines matched reported phenotypes. Ki-67-positive cells are shown.

interferon-gamma induced transcription factor interferon regulatory factor 1 (IRF1). We are deploying multiparameter phenotypic analysis in companion diagnostics development to identify the presence of surface protein drug targets on identified CTCs. This has the potential for immediate clinical utility and is not achievable by plasma nucleic acid analysis.

Application Beyond CTCs

Using similar methods of sample preparation, multiparameter staining, imaging and analysis, and cell retrieval, the platform has been applied to other rare cell investigations. Areas include fetal cell analysis for noninvasive prenatal genetic testing (17), minimal residual disease monitoring of liquid tumors, identification of rare immune cell subsets in blood, and pathological analysis of fine needle aspirate, other cytology samples, and tissue sections.

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