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High-throughput cytotoxicity and antigen-binding assay for screening small bispecific antibodies without purification

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The cytotoxicity of T cell-recruiting antibodies with their potential to damage late-stage tumor masses is critically dependent on their structural and functional properties. Recently, we reported a semi-high-throughput process for screening highly cytotoxic small bispecific antibodies (i.e., diabodies). In the present study, we improved the high-throughput performance of this screening process by removing the protein purification stage and adding a stage for determining the concentrations of the diabodies in culture supernatant. The diabodies were constructed by using an *Escherichia coli* expression system, and each diabody contained tandemly arranged peptide tags at the C-terminus, which allowed the concentration of diabodies in the culture supernatant to be quantified by using a tag-sandwich enzyme-linked immunosorbent assay. When estimated diabody concentrations were used to determine the cytotoxicity of unpurified antibodies, results comparable to those of purified antibodies were obtained. In a surface plasmon resonance spectroscopy-based target-binding assay, contaminants in the culture supernatant prevented us from conducting a quantitative binding analysis; however, this approach did allow relative binding affinity to be determined, and the relative binding affinities of the unpurified diabodies were comparable to those of the purified antibodies. Thus, we present here an improved high-throughput process for the simultaneous screening and determination of the binding parameters of highly cytotoxic bispecific antibodies.

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[**Key words:** High-throughput screening; T-cell recruiting antibody; Cytotoxicity screening; Binding affinity screening; Sandwich ELISA; Cancer therapy]

Antibodies with high target specificity and affinity are an important part of modern therapeutic and diagnosis approaches. Module-based platforms have been developed that allow recombinant antibodies to be constructed with specific structures and functions. Bispecific antibodies, which are fusion proteins reconstructed from two different antibodies that bind to different antigens, are promising therapeutic molecules. For example, the bispecific antibody ACE910 promotes blood clotting in patients with hemophilia A by simultaneously binding to Factor IXa and Factor X (1). Similarly, bispecific antibodies can induce immune responses by binding simultaneously to the surfaces of cancer cells and immune cells such as cytotoxic T cells. Because of their abundance and proliferative capacity, T cells are a potentially potent means of killing tumor cells (2–4). A previous report has also demonstrated a bispecific T-cell-recruiting antibody that can circumvent the immunological escape mechanism in tumors (5).

Compact T-cell-recruiting bispecific antibodies only reconstructed from fragments of antibody variable region (Fv) without constant region are well-studied constructs. These bispecific antibodies have low immunogenicity (6,7) and high penetration into tumor masses (8,9), and they can be produced by bacterial expression systems (10,11), which is a low-cost means of production. To date, a large number of compact T-cell-recruiting bispecific antibodies with different cancer targets and structures have been constructed and studied, and these studies have shown that the cytotoxicity of these constructs against cancer cells depends on the target antigen and epitope, and on the binding affinity and structure of the bispecific antibody (12); changing the target cancer cell antigen or replacing the anti-cancer Fv with another Fv with affinity for the same target can produce as much as a 10^3-fold difference in cytotoxicity (13–15). Therefore, when constructing highly cytotoxic bispecific antibodies, suitable Fvs for the structural format should be used; however, how the binding properties of the Fvs used in the design of bispecific antibodies affects their cytotoxicity remain to be elucidated.

Recently, we proposed a semi-high-throughput process for screening the cytotoxicity of bispecific antibodies (15). In this previous study, more than 100 T-cell-recruiting bispecific antibodies with a diabody format, where a pair of single-chain Fvs (scFvs) with swapped variable regions of heavy-chain (V_H) and light-chain (V_L) domains are dimerized (16), were prepared by using an *Escherichia coli* expression system, and the cytotoxicity of these diabodies against cancer cells was rapidly and quantitatively determined.

In the present study, we improved the high-throughput performance of our previous process by removing the purification stage and adding a stage for determining the concentrations of the

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diabodies in culture supernatant. Our previous method showed that the cytotoxicity of the unpurified diabodies against cancer cells was able to be qualitatively assayed with the culture supernatant of the transformed *E. coli*, but a purification stage was needed to quantify cytotoxicity (15). In the present study, we improved the previous process by using a tag-sandwich enzyme-linked immunosorbent assay (ELISA) technique to detect the diabody of the culture supernatant without purification stage. The assistance of tag-sandwich ELISA enabled to measure the cytotoxicity of the diabodies with comparable accuracy to the previous process. Furthermore, we incorporated an assay for determining the binding affinity of the diabodies to target cells, because binding affinity is a crucial factor underlying the cytotoxicity of T-cell-recruiting diabodies (15). Although contamination of the culture supernatant prevented quantitative determination of binding affinity by means of a surface plasmon resonance (SPR) approach, relative binding affinity values were determined and the dissociation behavior of the unpurified diabodies was comparable to that of the purified antibodies. Thus, we demonstrate an improved high-throughput method for simultaneously screening and determining the binding parameters of highly cytotoxic antibodies (Fig. 1).

MATERIALS AND METHODS

Preparation of purified diabodies Diabodies with c-Myc tag and polyhistidine tag at the C-terminus and affinity for both epidermal growth factor receptor (EGFR) and either CD3 or CD28 were prepared by transforming BL21 Star (DE3) *E. coli* with expression vectors constructed previously (15). The transformed cells were grown overnight at 28°C in LB broth supplemented with 100 μg/mL ampicillin, and then 5 mL of the cell culture was inoculated into 500 mL of 2× YT broth supplemented with 100 μg/mL ampicillin. Once the optical density of the culture medium had reached 0.8, isopropyl-1-thio-L-D-galactopyranoside was added to the flask to a final concentration of 0.1 mM to induce diabody expression, and the cells were cultured overnight at 20°C. Culture supernatant containing diabodies was removed from the cells by centrifugation at 12,000 ×*g* for 30 min, and the obtained diabodies were purified by means of immobilized metal affinity chromatography (IMAC) and size-exclusion chromatography (Hiload Superdex 200 prep-grade column; GE Healthcare, IL, USA). The concentration of each purified diabody was determined by the absorbance at 280 nm by using an ultraviolet spectrophotometric method and the molar extinction coefficient.

Preparation of culture supernatant for high-throughput screening Transformed cells were grown overnight at 28°C in LB broth supplemented with 100 μg/mL ampicillin in a deep-well plate (Axygen, CA, USA). Then, 120 μL of the cell culture was inoculated into 1080 μL of 2× YT broth supplemented with 100 μg/mL ampicillin in a deep-well plate. After incubating for 2 h at 28°C, isopropyl-1-thio-L-D-galactopyranoside was added to each well to a final concentration of 0.1 mM to induce the diabody expression, and cells were incubated for a further 10 h at 20°C. The culture supernatant was separated from the cells by centrifugation at 3000 ×*g* for 30 min, and they were sterilized by filtration by using a MILLEX GV 0.22 μm syringe filter unit (Merck, NJ, USA).

Tag-sandwich ELISA Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.5/150 mM NaCl) and a 150-fold dilution of a commercial solution of anti-c-Myc-tag antibody (final concentration, 14 nM; 9B11; Cell Signaling Technology Japan, Tokyo, Japan) were incubated in the wells of a 96-well polystyrene ELISA microplate (655061; Greiner, Austria) for 1 h, and then 0.5 w/v% bovine serum albumin in TBS was added to the wells. After washing each well with 0.05% Tween 20 in TBS, diluted culture supernatants from transformed *E. coli* were incubated for 30 min. The wells were washed again with 0.05% Tween 20 in TBS, and then incubated for 40 min at 25°C with a 2500-fold dilution of a commercial

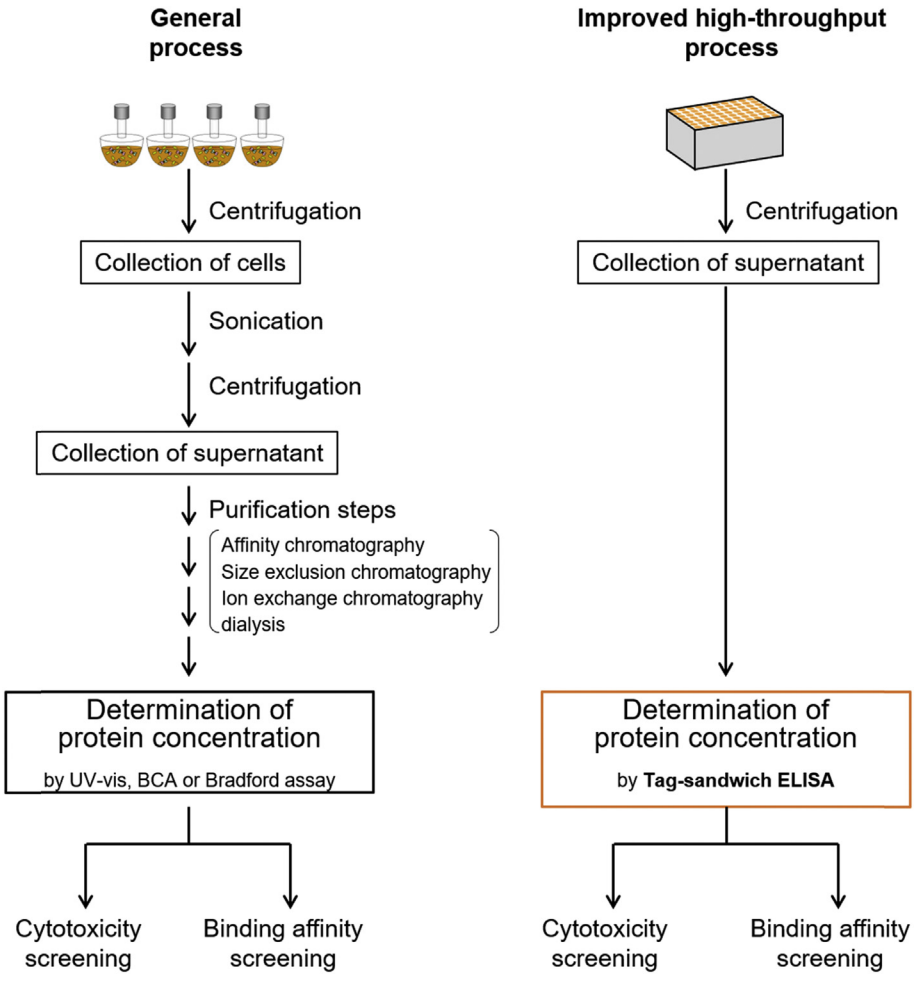

FIG. 1. General and improved high-throughput process for examining the cytotoxicity and antigen-binding of small bispecific antibody.

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