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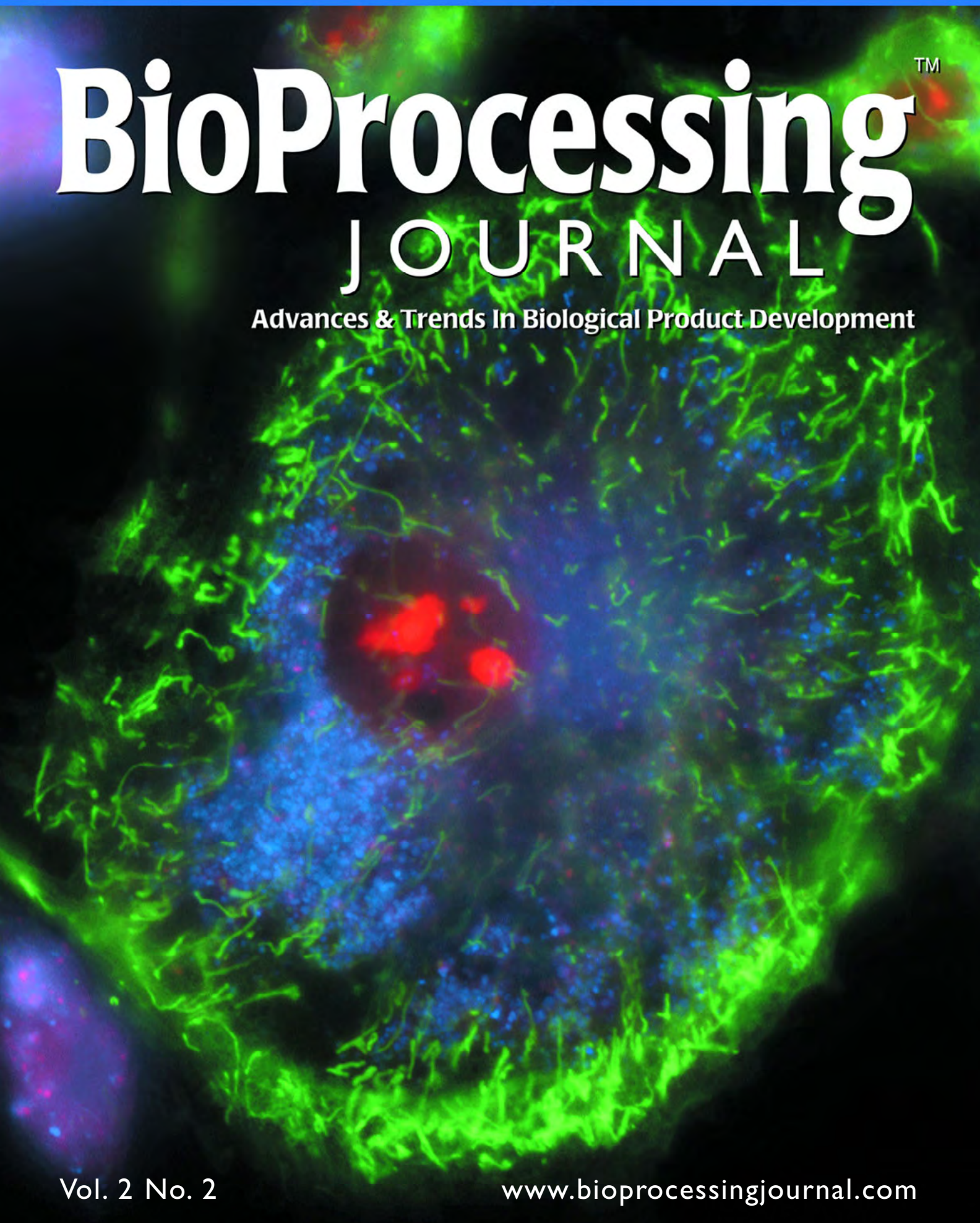

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Transfected Cell Line Enrichment Using the Gel Microdrop (GMD) Secretion Assay

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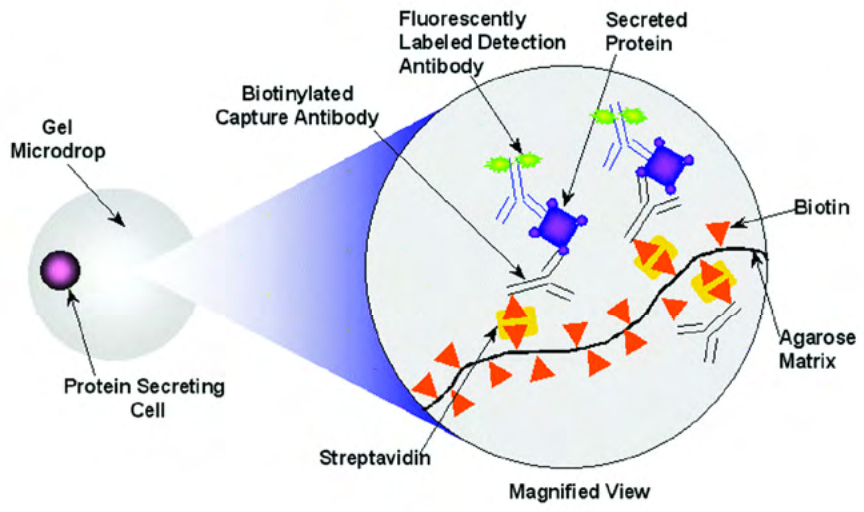

Figure 1. Schematic representation of the GMD Secretion Assay (details in the text)

The Gel Microdrop (GMD) Secretion Assay involves encapsulating cells within a biotinylated agarose matrix, followed by capture and detection of cell-secreted molecules with fluorescent markers. This technology differs from other encapsulation methods in that the small size of the microdrop (<50 µm diameter) creates a defined microenvironment around the cell without impeding diffusion of nutrients, antibodies, or nucleic acid probes into the GMDs, or the diffusion of secreted products out of the GMDs. Large numbers of GMDs can be readily analyzed using flow cytometry, and sub-populations of rare or high-secreting cells, as small as 0.1%, can be detected and recovered in one day. This assay format is a rapid alternative to limiting dilution cloning (LDC).

Using flow cytometry, encapsulated cells are analyzed or recovered, based on the amount of protein captured in each GMD. The capture and detection is analogous to the sandwich antibody format used in enzyme-linked immunosorbent assays (ELISA), however, the immunoreaction itself takes place within the agarose microsphere, leaving the encapsulated cell intact. Viable cells of interest can be recovered after sorting for use in generating cell lines for clinical research and immunotherapy. In this study, we demonstrated the effectiveness of the GMD Secretion Assay for analyzing heterogeneous populations of freshly transfected CHO cells, and for isolating high secretors by fluorescence activated cell sorting (FACS).

Introduction

The commercial importance of genetically modified or transfected cells for producing therapeutic proteins is well established. Obtaining and characterizing a pure population of transfected cells, or evaluating the efficacy of different transfection methods, usually requires inclusion of a drug resistance gene in a vector and the subsequent clone selection, which use time consuming and labor intensive limited dilution cloning (LDC). Ordinarily, at least two rounds of cloning are required in order to generate productive clones.[1-3] Other experimental systems have been developed to enrich cell lines for high secretors, including flow cytometric detection based on cell surface immunoglobulin expression,plus analysis and sorting of live cells based on identification of secreted molecules bound to a cell surface affinity matrix.[4-8] To evaluate genetic stability and functional phenotype of cell lines generated for bioprocessing, you must determine the copy number of the genetic elements encoding the antibody heavy and light chains, or assess the intracellular levels of antibody with fluorescently labeled anti-IgG antibodies.[9,10] These methods can provide valuable information about individual cells within a heterogeneous cell population, such as the genetic or phenotypic (functional) instability. However,

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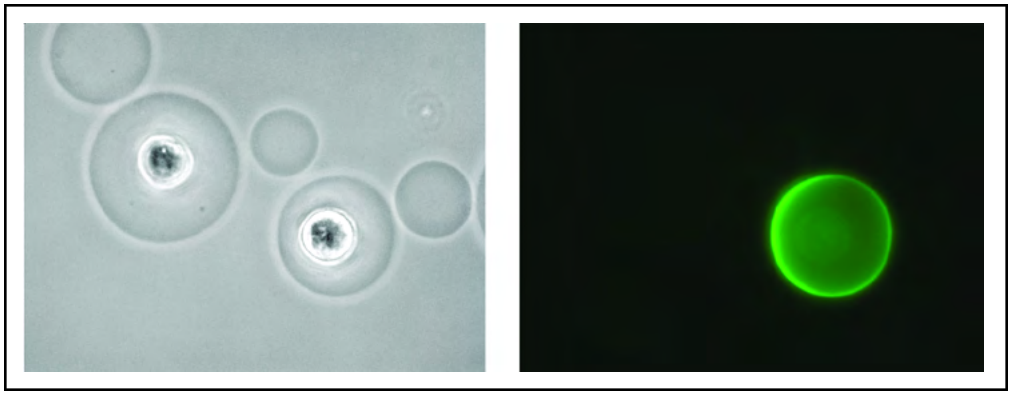

Figure 2. Discrimination of secreting and non-secreting cells using the GMD Secretion Assay. On the left, two GMDs occupied by CHO cells and unoccupied GMDs are shown using phase-contrast microscopy. However, on the right, only the GMD occupied by a secretor cell exhibits green fluorescence after labeling secreted product with goat anti-mouse IgG1 specific FITC conjugated Ab.

practical limitations of existing methods suggest the need for a rapid, simple, and sensitive technique that can select for high producing cell lines.

In this study, we used the GMD Secretion Assay to analyze, discriminate, and isolate individual secretors from heterogeneous populations of transfected CHO cells, based on level of secreted fusion protein with anti-hTNFα activity. Recombinant proteins are extensively used in basic research and bioprocessing. The GMD Secretion Assay format has previously been used to measure immunoglobulin secretion from individual hybridoma cells, plus recombinant protein secretion by transfected cell lines.[3,11-16]

GMD technology makes it possible to encapsulate single cells into agarose microspheres, and then use flow cytometry to measure recombinant protein secretion from individual cells. As with microtiter well and Petri dish inoculation, Poisson statistics adequately describes the frequency at which individual cells are encapsulated within GMDs formed with a low concentration of cells ($2x10^6$). To obtain GMDs with a high probability of containing zero or one cell, the cell concentration is adjusted so that approximately 7–10% of GMDs are occupied, in a manner analogous to LDC. Thus, GMD technology effectively separates a cell population into individual components, and in conjunction with flow cytometry, permits discrimination and isolation of GMDs containing cells of interest. The GMD Secretion Assay makes it possible to use the inherent power of flow cytometry, including light scatter gating and multiparameter fluorescence analysis, to rapidly discriminate, isolate, and recover individual cells of interest on the basis of secretion. For this application, sub-population analysis permits discrimination of unoccupied GMDs from those occupied by either more than a single cell or by non-viable cells. GMDs containing single viable cells that are secreting a protein of interest can be identified, and the amount of the secretion can be quantified on the basis of GMD-associated fluorescence. Cells of interest, such as high secretors, can then be isolated using FACS. The ability to capture secreted protein and quantify fluorescence at the individual cell level is a compelling feature of the GMD Secretion Assay. Since the assay can be performed in four to five hours, individual cell secretion of recombinant proteins can be analyzed, and cells of interest can be isolated in a single day.

While performing the GMD Secretion Assay, there is a concern about the theoretical possibility of generating false-positive GMDs due to "crosstalk," or leakage of the secreted product from GMDs occupied by secretors. "Crosstalk" can be virtually eliminated by choosing the appropriate secretion time. For example, when performing the GMD Secretion Assay to isolate high protein secreting CHO and hybridoma cells for bioprocessing applications, cells can be sorted as soon as the signal exceeds background, and before the antibody binding sites can become saturated. Quantitative secretion analysis and inter-experiment comparisons can also be performed to ensure that the number of capture sites exceeds the number of secreted molecules.

A schematic representation of the GMD Secretion Assay is shown in Figure 1. In this assay, cells are encapsulated in an agarose matrix to which biotin has been covalently bound. Addition of streptavidin (SA) forms a bridge between the biotinylated agarose and the biotinylated capture Ab. During the secretion incubation step, secreted product diffuses through the gel matrix and binds to the biotinylated Ab capture sites. Fluorescently labeled Abs are added to the surrounding medium, where they diffuse into the GMD matrix, bind to the captured secreted protein, and label the GMDs occupied by secretors. The possibility of discriminating recombinant protein secreting cells from non-secretors, or cells with high secretion level from low secretors, is a significant advantage of the assay. As shown in Figure 2, FITC labeled detection Ab is bound to the agarose matrix of GMDs occupied by the protein-secreting cell. GMDs occupied by non-secreting cells are not labeled by detection Ab. Mean fluorescence intensity (MFI) of occupied GMDs is then quantitated by flow cytometry. Figure 3 shows the proportional increase in MFI during the secretion incubation. After immobilizing SA and biotinylated capture Ab in the agarose matrix, and and then incubating encapsulated transfected CHO cells in growth medium at 37° C and 5% CO_2, the increase in MFI correlates with the duration of the secretion incubation. In other words, the longer the incubation time, the higher the MFI.

The GMD Secretion Assay format, when combined with flow cytometry, makes this technology a powerful tool for rapid isolation of viable single cells based on the level of protein secretion. After isolating cells of interest by FACS, a low concentration (1-5 U/ml) of agarase is used to digest the matrix, and the cells can be expanded in culture or cloned after incubating in growth medium.

Materials and Methods

Cells and culture conditions

Freshly transfected (one week after selection),Flp-In CHO cells (Invitrogen) were used for the experiments. The cDNA encoding the extracellular domain of the human TNF-α receptor p75 chain was PCR amplified from a cDNA library of human PBMCs activated by LPS. The PCR primers were designed according to the published p75 sequence (Genbank). The 5' primer contains a Pci restriction site, while the 3' primer contains a Xho I restriction site. After digestion of PCR products with Pci and Xho I, the insert was ligated into a mammalian expression vector that had been similarly treated. This vector was derived from pSecTag/FRT/V5-His-TOPO (Invitrogen), and was inserted in a human IgG Fc cassette (by Dr. W. Gao, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA). The p75 sequence was fused in-frame with IgG Fc, which contains a Hisx6 tag at the C-terminal end. According to the manufacturer's instructions, a single clone of the ligated product (1.0 µg) was co-transfected with pOG44 (10.0 µg, Invitrogen) into the Flp-In CHO cell line (Invitrogen) using GeneJammer transfection reagent (Stratagene). Twenty-four hours later, transfected cells were subjected to drug selection with 800 µg/ml of Hydromycin B in UltraCHO medium (BioWhittaker), that was supplemented with 5% FBS. Drug resistant colonies appeared after about two weeks of selection. Cells constitutively secreting recombinant fusion protein (Fc portion of hIgG combined with hTNFαR) were maintained in UltraCHO medium without serum, and were subcultured every three to four days by splitting 1:10.

Evaluation of immunoreagents using unoccupied GMDs

We first evaluated immunoreagents used in the GMD Secretion Assay format by generating titration curves with purified human IgG (Fc portion). Unoccupied GMDs made with CBG3-SA™ were incubated with biotinylated anti-human IgG (Fc portion specific), washed with HBSS, and counted in a hemocytometer chamber. Aliquots of the GMD suspension ($1x10^6$ GMDs per sample) were incubated with purified hIgG (Fc portion) at concentrations ranging from 1250.0 to 2.4 ng/ml. Then, GMDs were washed in HBSS, incubated with FITC conjugated anti-human IgG (Fc portion specific), washed again. Finally, the mean fluorescence was analyzed by flow cytometry using a FACScan.

GMD encapsulation of transfected CHO cells

For encapsulation, 500 µl of biotinylated agarose (CBG-3™, One Cell Systems, Inc.) was melted in a microwave for 75 seconds and mixed with 25 µl of 10% pluronic acid (Sigma). After adjusting the temperature to 37° C, 120 µg of SA (Sigma) and $2x10^6$ transfected CHO cells in 100 µl of HBSS, were added to the agarose matrix. The agarose-SA-cell mixture was then added to 15 ml of sterile CelMix™ (One Cell Systems, Inc.) at 37° C, and the sample was emulsified at 2,100 rpm for one minute at room temperature (RT), one minute on ice, and at 1,100 rpm for six minutes on ice using the CellSys-100™ Microdrop Maker (One Cell Systems, Inc.). GMDs were separated from CelMix™ by transferring the emulsion to two 15 ml Falcon tubes, washing with HBSS, and centrifuging at 1,800 rpm for 10 minutes. The pellet was then transformed to a test tube, resuspended in HBSS, centrifuged at 1,500 rpm for five minutes, and resuspended in 4 ml of HBSS. After filtering through a sterile 40 µm cell strainer (Falcon),GMD occupancy and cell viability were evaluated using Trypan Blue staining and phase contrast microscopy. One 0.3 ml aliquot of GMD suspension was saved for the negative (no capture Ab) control.

Immunoassay for recombinant Ab

CromPure human IgG (Fc fragment), plus biotinylated and FITC-labeled goat anti-human IgG (Fc fragment specific) were obtained from Jackson ImmunoResearch Laboratories. Eighty µg of capture (biotinylated) Ab was resuspended in 4 ml of HBSS,mixed with 4 ml of the GMD suspension containing encapsulated cells, and incubated for 12 minutes at RT on a rocking platform. After incubation, the GMD suspension was washed two times with HBSS supplemented with 2.5% FBS by centrifugation at 1,500 rpm for five minutes. The pellet was resuspended in 3 ml of Ultra CHO medium, and two 0.3 ml aliquots were saved for positive and supernatant controls. Encapsulated cells were added to the flask containing 30 ml of Ultra CHO medium and incubated for 90 minutes (37° C and 5% CO_2) in order to permit antibody secretion. After incubation, the secretion sample and controls were centrifuged at 1,500 rpm for five minutes, and pellets were resuspended in 0.5 ml (controls) or in 1.5 ml (secretion sample) of HBSS supplemented with 2.5% FBS. FITC-conjugated Ab at 10 µg/ml in HBSS+2.5% FBS were then added to controls (0.5 ml) and secretion sample (1.5 ml),and then incubated for 20 min at RT on a rotating platform. After washing two times with

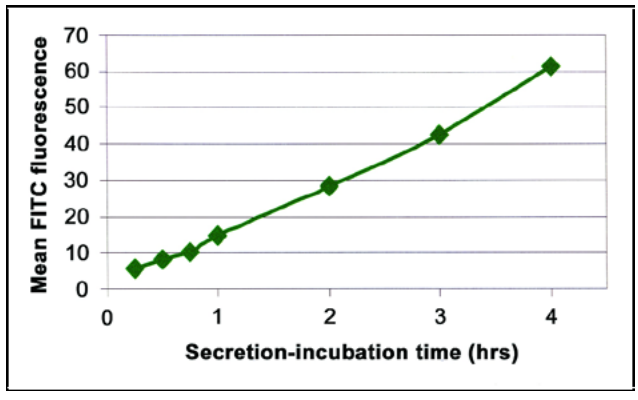

Figure 3. Kinetics of mIgG1 secretion by encapsulated transfected CHO cells. The GMD Secretion Assay was performed with a transfected CHO cell clone that secreted mouse IgG1. Approximately $1x10^6$ cells were encapsulated in CelBioGel-3™ and incubated at 37° C and 5% CO_2 to permit Ab secretion. FITC MFI associated with the labeled secreted product was measured with a FACScan after incubating the secretion sample for different periods of time from 15 to 240 minutes. The increase in MFI correlated with increased secretion incubation time.

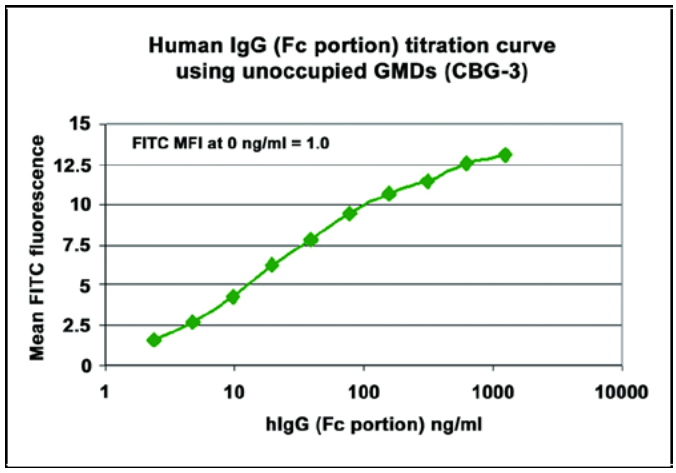

Figure 4. Titration curve of purified human IgG (Fc portion) using unoccupied GMDs.

HBSS+2.5%FBS, pellets were resuspended in Ultra CHO medium and transferred to tubes for flow cytometric analysis. In addition to the secretion samples, three controls were included in each GMD Secretion Assay: 1) negative control (no capture Ab), 2) positive control (purified hIgG, Fc portion at 1 µg/ml), and 3) an internal control with cell culture supernatant collected on the day of the experiment.

Flow cytometry analysis and FACS

Flow cytometric analysis and sorting were performed with a FACScan (Becton Dickinson) and an EPICS Elite (Coulter Corp.) equipped with a 100 µm quartz nozzle. FITC-labeled detection Abs were excited by the 488 nm spectral line of an argon ion laser. Green (FITC) fluorescence was detected using a 525 nm band pass filter. For acquisition and statistical analysis, EPICS Elite 4.02 software was used. Occupied GMDs were distinguished from unoccupied GMDs by increased forward (FSC) and right angle light scatter (SSC). The light scatter properties of occupied GMDs were easily resolved from those of unoccupied GMDs, which were eliminated from the data acquisition by increasing the forward scatter threshold or discriminator until unoccupied GMDs did not appear on the screen (Fig. 5, dot plot 1). Subpopulations of multiple occupied GMDs (which have higher FSC characteristics) and GMDs occupied by dead cells (high SSC) were not included in the data acquisition gate. Background fluorescence was determined with no capture control. For flow cytometric analysis, samples were run at a rate of approximately 600 events per second with 10,000 or more gated events collected. FITC fluorescence higher than the negative control represented antibody-secreting cells. Sub-populations of the brightest occupied GMDs were gated and sorted at a rate of at least 10^6 GMDs per hour. Approximately 50,000 events were collected.

Cell recovery after sorting

After sorting, isolated GMDs were pooled and placed in 1 ml of Ultra CHO medium supplemented with agarase (1 U/ml), and then incubated at 37° C and 5% CO_2 until the cell density reached $2x10^6$. Then, cells were transferred to a 15-ml flask (usually after incubating for seven to 10 days). After expansion, cells were re-enriched by performing a second round of the GMD Secretion Assay, and then cloned or maintained in culture as described above.

Results and Discussion

Evaluation of immunoreagents using unoccupied GMDs

The titration curve shown in Figure 4 revealed a proportional increase in FITC MFI, which correlated with hIgG (Fc portion) concentration. Background fluorescence in the sample incubated in HBSS alone (negative control, no hIgG added) had significantly lower fluorescence than the sample incubated with 4.8 ng/ml of tested hIgG (MFI was 1.0 vs 2.7, respectively). Therefore, the detection level of the assay was between 4.8–9.7 ng/ml, which corresponds to 4.8–9.7 fg/GMD ($1x10^6$ GMDs/sample). These results indicate the sensitivity of the GMD Secretion Assay, and the ability to use flow cytometry to discriminate GMDs occupied by a single cell secreting approximately 10 or more femtograms (fg) of recombinant protein during the secretion incubation time. Similar to results using a conventional solid phase ELISA, the concentration of the secreted product in a cell culture supernatant can be determined by extrapolating MFI from the supernatant control. This internal control is generated by incubating an aliquot of GMD suspension with neat or diluted cell culture supernatant collected on the day of the experiment. Thus, the supernatant control provides additional information about the functional (secretory) activity of cells in bulk culture prior to analyzing individual cells with the GMD Secretion Assay.

GMD Secretion Assay using freshly transfected CHO cells

Analysis of the secretory activity of individual cells, and the ability to isolate secretors at the early stages of cell line development, is important for evaluating the efficiency of different transfection procedures, enriching for rare cells or high secretors, and reducing the time required for developing highly productive clones. Shortly after transfection, and prior to cloning, recombinant cell lines are usually comprised of a substantial proportion of non-secreting cells. A sub-population of phenotypically non-productive cells, which can be genetically identical to the producer line, also arises during the scale-up process. Later, this non-producing population can represent a majority of the cells in culture, resulting in a decrease in overall cell line productivity. To address these issues, we investigated if the GMD Secretion Assay, when combined with FACS, could be used to select secretors from a heterogeneous population of freshly transfected cells (one week after selection). Using freshly transfected CHO and NSO cell lines (NSO data not shown in this report), results were similar and support the use of the GMD Secretion Assay for rapid cell line enrichment.

In this study, we demonstrated that the GMD Secretion Assay can be successfully used to analyze and detect recombinant Ab or protein secretors from a heterogeneous population of freshly transfected,uncloned CHO cells. After transfection, and as described in Materials and Methods, Flp-In CHO cells were grown for two weeks under selection pressure. Recovered cells were maintained in growth medium (Ultra CHO) for one week, and then the level of hIgG (Fc portion) of the recombinant fusion protein (with anti-hTNFα activity) secreted by individual cells was quantitated using the GMD Secretion Assay and FACS. The first round of enrichment was performed after encapsulating $2x10^6$ cells. A low level of background fluorescence (MFI = 0.178) was detected in the negative control (no capture Ab) (Fig. 5, histogram 2). This control, which is generated by incubating an aliquot of encapsulated cells with fluorescently labeled detection Ab, is used to assess the level of autofluorescence, and then determine if the protein of interest is also expressed on the cell surface. Based on these results, a marker is established with flow cytometric analysis for identifying positive events with higher fluorescence than in the negative control. FITC MFI detected in the positive control (1 µg/ml of purified Fc portion of hIgG) was 3.58 (Fig. 5, histogram 3). As expected, the overall productivity of the entire population of freshly transfected CHO cells was low. FITC MFI in the supernatant control was 0.58, and was generated by incubating

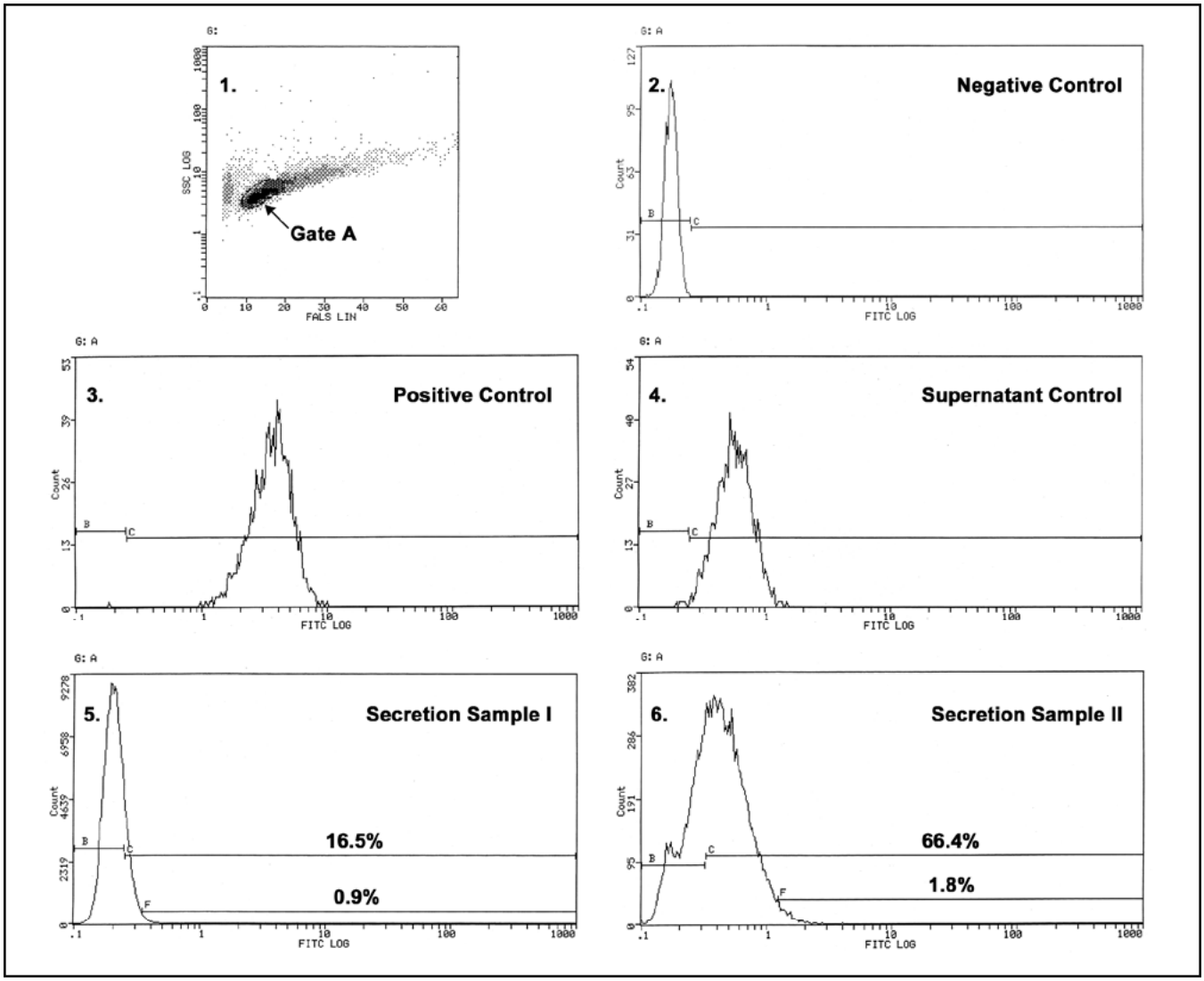

Figure 5. Enrichment of freshly transfected CHO cells secreting a hFc-hTNFαR fusion protein using the GMD Secretion Assay. A first round of enrichment using the GMD Secretion Assay was performed after encapsulating $2x10^6$ freshly transfected CHO cells. GMDs occupied with single, viable cells were gated (dot plot 1, gate A). Flow cytometric analysis of the negative, positive, supernatant controls, and the secretion sample after a two hour incubation was performed (histograms 2,3,4, and 5, respectively) using EPICS Elite to measure FITC MFI. 16.5% of positive cells were detected in the secretion sample (histogram 5, marker C). A 0.9% sub-population of GMDs exhibiting the highest fluorescence (histogram 5, marker F) were sorted and expanded in culture. After expansion, cells were re-encapsulated and analyzed using the GMD Secretion Assay. Secretory profile of the sample from the second round of enrichment is shown on histogram 6. After the first round of enrichment, the percentage of secretors increased from 16.5% to 66.4% (marker F, histograms 5 and 6, respectively). A 1.8% sub-population of the highest secretors (histogram 6, marker F) was sorted and expanded in culture.

one aliquot of the GMD suspension with undiluted cell culture supernatant collected on the day of the experiment (Fig. 5, histogram 4). Based on these results, the concentration of the secreted recombinant Ab in the cell culture supernatant was approximately 160 ng/ml.

A sub-population of GMDs occupied by single, viable cells was gated and then used to determine FITC fluorescence (Fig. 5, dot plot 1, gate A). After a two hour secretion incubation during the first round of enrichment, 16.5% of the GMDs were occupied by single viable cells secreting fusion protein that contained the Fc portion of hIgG (Fig. 5, histogram 5, marker C). FITC MFI of this sub-population was 0.28. A 0.9% sub-population of secretors exhibiting the highest fluorescence was sorted (marker F). Isolated cells were resuspended in 1 ml of growth medium supplemented with 1U/ml of agarase and then incubated at 37° C and 5% CO_2. After expanding in culture for 10 days, $2x10^6$ cells were re-encapsulated and a second round of enrichment was performed with the GMD Secretion Assay. The supernatant control revealed increased cell line productivity. The FITC MFI was 1.82, or approximately 500 ng/ml (data not shown), versus a MFI of 0.58 (approx. 120 ng/ml) detected during the first round of enrichment. After a two hour secretion incubation, analysis of the secretion sample showed that a significantly higher proportion of hIgG cells secreted fusion protein containing the Fc portion (66.4% vs 16.5% during the first round of enrichment). FITC MFI of this sub-population was 0.55, which is 1.96 times higher than the MFI of the sub-population of secretors identified during the initial round of FACS enrichment (0.55 vs 0.28, respectively). These results demonstrate that the GMD Secretion Assay can be used as an alternative to LDC to identify the number of positive cells in culture, and to determine the level of recombinant protein secreted by individual cells. In addition, the cell line can be enriched for high secretors. After the first round of enrichment using the GMD Secretion Assay, results show that the concentration of the secreted product in cell culture supernatant was approximately 4.16 times higher than the concentration measured prior to enrichment (500 ng/ml vs 120 ng/ml, respectively), when using identical cell culture conditions. FITC MFI of GMDs occupied by single secretors was also 1.96 times higher, and demonstrated increased cell productivity. To further enrich the cell population for high secretors, 1.8% of GMDs exhibiting the highest fluorescence (MFI 1.54) were sorted and expanded (37° C and 5% CO_2) in Ultra CHO medium supplemented with 1U/ml of agarose. After incubating for seven days, the cell concentration was $5x10^5$/ml, which was similar to the density obtained in the previous round of enrichment. As described above, the amount of secreted protein in the cell culture supernatant, when diluted 1:2, was tested using $1x10^6$ unoccupied GMDs. Based on FITC MFI of 2.12, the concentration of recombinant fusion protein in the cell culture supernatant was estimated to be <1 µg/ml, which indicates that, after enrichment, the cell population included a significantly higher proportion of high secretors.

Conclusions

The GMD Secretion Assay, combined with FACS, represents a rapid, quantitative alternative to limiting dilution cloning (LDC). In this report, we showed that the GMD Secretion Assay could be used to analyze a heterogeneous cell population and isolate cells of interest (such as high secretors) in a single day. After enrichment for high secretors using the GMD Secretion Assay and FACS, cell line productivity increased. These studies also showed that encapsulation, assay for immunofluorescence, and sorting did not have adverse effects on viability or secretory activity of cells (data not shown). In addition, encapsulated cells isolated by FACS can be rapidly recovered and expanded in culture or cloned. The data demonstrate that by monitoring the population of secretors in a heterogeneous cell population, the GMD Secretion Assay can be successfully used at early stages of cell line development to enrich cultures for high secretors, eliminate low producers, evaluate the effectiveness of different transfection techniques and reagents, and evaluate the functional phenotype (secretory activity) of genetically stable transfected cell lines.

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