

# Automated In Situ Measurement of Cell-Specific Antibody Secretion and Laser-Mediated Purification for Rapid Cloning of Highly-Secreting Producers

Elie G. Hanania, Annabeth Fieck, Janine Stevens, Leon J. Bodzin, Bernhard Ø. Palsson, Manfred R. Koller

Cyntellect, Inc., San Diego, California; telephone: (858) 550-1770; fax: (858) 550-1774; e-mail: fkoller@cyntellect.com

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**Abstract:** Cloning of highly-secreting recombinant cells is critical for biopharmaceutical manufacturing, but faces numerous challenges including the fact that secreted protein does not remain associated with the producing cell. A fundamentally new approach was developed combining in situ capture and measurement of individual cell protein secretion followed by laser-mediated elimination of all non- and poorly-secreting cells, leaving only the highest-secreting cell in a well. Recombinant cells producing humanized antibody were cultured serum-free on a capture matrix, followed by staining with fluorescently-labeled anti-human antibody fragment. A novel, automated, high-throughput instrument (called LEAP™) was used to image and locate every cell, quantify the cell-associated and secreted antibody (surrounding each cell), eliminate all undesired cells from a well via targeted laser irradiation, and then track clone outgrowth and stability. Temporarily sparing an island of helper cells around the clone of interest improved cloning efficiency (particularly when using serum-free medium), and helper cells were easily eliminated with the laser after several days. The in situ nature of this process allowed several serial sub-cloning steps to be performed within days of one another, resulting in rapid generation of clonal populations with significantly increased and more stable, homogeneous antibody secretion. Cell lines with specific antibody secretion rates of >50 pg/cell per day (in static batch culture) were routinely obtained as a result of this cloning approach, often times representing up to 20% of the clones screened. © 2005 Wiley Periodicals, Inc.

**Keywords:** recombinant antibody production; protein production; high-producing clones; cell culture; process development; cell cloning

## INTRODUCTION

The rapidly increasing number of therapeutic proteins entering clinical development and use demands improved methods to support rapid and reliable development of efficient recombinant protein manufacturing processes.

Correspondence to: Manfred R. Koller

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There is a current and projected shortage of worldwide GMP manufacturing capacity for therapeutic proteins, and new approaches for shortening timelines and increasing product yield from the available bioreactor capacity are deemed critical (Ransohoff et al., 2004; Walsh, 2003). A major bottleneck in biopharmaceutical process development is the generation of stable highly-secreting production cell lines.

Cloning of cells for commercial production is typically performed by serial limiting dilution, an approach that is inefficient and time consuming, placing cloning into the critical path of commercial process development. Protein secretion on an individual cell basis (i.e., specific protein secretion) cannot be measured by limiting dilution, requiring outgrowth of a clone prior to measurement of average productivity (e.g., by ELISA of the culture supernatant) and subsequent selection. This limitation precludes direct selection of the best cell in a population, necessitates that substantial cell numbers be generated before the desired property (i.e., protein secretion) can be measured, and usually requires a significant number of long, serial sub-cloning steps (Underwood and Bean, 1988). Finally, cloning efficiency can be low with limiting dilution, and wells initially receiving more than one cell usually have a growth advantage due to medium conditioning effects (Underwood and Bean, 1988). Therefore, limiting dilution approaches generally require use of richer medium (i.e., serum-containing) versus that used in bioreactor production to improve cloning efficiencies, obligating another downstream step for adaptation of clones to production conditions.

Flow cytometry, a commonly used approach for cell purification, cannot be used to directly detect secreted cell products that are not cell-associated. To overcome this limitation, a gel microdrop encapsulation method has been developed to allow capture of secreted protein and subsequent cell sorting by flow cytometry (Atochina et al., 2004; Gray et al., 1995; Powell and Weaver, 1990). This approach to cell cloning adds the complexity of encapsulating the cells,

verifying the contents (i.e., clonality) of each capsule, and then recovering the single cells of interest from the capsules. A similar approach has been described using an artificial affinity matrix directly cross-linked on the cell surface within a low permeability medium (gelatin-based) (Manz et al., 1995). A drawback of this latter approach is that its low saturation limit is reached in less than an hour (Holmes and Al-Rubeai, 1999), which is problematic given that antibody production varies during the cell cycle (Cherlet et al., 1995). Furthermore, both of these flow cytometry-based approaches cannot be applied to small cell numbers, again necessitating significant clonal outgrowth between serial sub-cloning steps, all of which has been described as “user unfriendly” (Holmes and Al-Rubeai, 1999) and “difficult and time consuming” (Brezinsky et al., 2003). Consequently, development of production cell lines remains a non-automated, time consuming, and costly endeavor, generally requiring 4–8 months of effort. An improved method for rapid and reliable cloning of production cell lines therefore remains a key goal to improve the efficiency of process development for biopharmaceutical production.

Cyntellect has developed a novel technology, called LEAP<sup>TM</sup> (Laser-Enabled Analysis and Processing), for automated, high-throughput cell imaging coupled with laser-mediated cell manipulation. LEAP, which is based on semiconductor manufacturing equipment, provides significant advantages over microscope-based platforms with respect to throughput, robustness, and automation. Descriptions of LEAP can be found in prior publications and patents (Koller et al., 2003, 2004), and details regarding multi-color imaging and cell purification on this system have been published (Koller et al., 2004).

## MATERIALS AND METHODS

### Cells and Media

Cells were purchased from ATCC (Manassas, VA). A hybridoma cell line (172-12A4) producing IgG specific for the human *v-erb* B oncogene product was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (Invitrogen; Carlsbad, CA). An NS0 cell line (20C2) producing IgG specific for the p75 heterodimer of human IL-12 was originally cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 0.1 mM 2-mercaptoethanol and 10% FBS, and was later adapted to serum-free medium (HyQ-SFM4MAB-NS0; Hyclone; Logan, UT). A CHO cell line (B13-24) secreting humanized IgG against the beta chain (CD18) of human leukocyte integrin was originally cultured in IMDM supplemented with 10% FBS, and was later adapted to serum-free medium (HyQ-SFM4MAB-CHO-Utility, Hyclone).

### In Situ Capture and Staining of Secreted Protein

Cells were plated in serum-free medium at 4,000–8,000 (or 200–400) per well in 96-well (or 384-well) plates

(Cytellect; San Diego, CA) in the presence of protein G (10–30 µg/mL; Amersham; Piscataway, NJ) and blocked with bovine serum albumin (BSA; 0.1% in growth medium; Sigma; St. Louis, MO). After 6–48 h of incubation at 37°C, wells were further blocked with fetal bovine serum (FBS; 5% in growth medium) and washed several times with serum-free medium. A PE-conjugated, affinity-purified goat anti-human IgG F(ab')<sub>2</sub> fragment (Rockland; Gilbertsville, PA) was added at 1:200 dilution and CellTracker Green (Invitrogen) was added at 5 µM to each well. After 1 h of incubation at 4°C, wells were washed several times with serum-free medium and plates were imaged and processed on LEAP.

### Cell Detection, Protein Measurement, and Cloning on LEAP

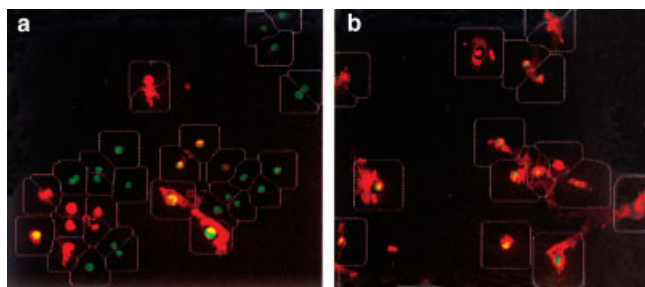
Following the capture and staining of secreted antibody, LEAP was used to identify highly-secreting cells and eliminate unwanted non- and poorly-secreting cells from each well. LEAP automatically performed an auto-focus operation at the center of each well and then captured 36 pairs (green and red fluorescence) of 5 × images covering the entire area of the well (using rapidly turning galvanometer mirrors with the plate held stationary), a process that was repeated for each well of a 96-well plate. For 384-well plates, one auto-focus operation was performed in the center well of a nine-well area and then 36 pairs (green and red fluorescence) of 5 × images were captured covering the nine wells, a process that was repeated 48 times to cover the plate. Custom software algorithms were developed to automatically locate all cells based on green fluorescence, quantify cell-associated and secreted antibody based on red fluorescence, and identify desirable clones based on multiple criteria. After imaging and image processing of an area as described above, but before the plate was moved to the next position, the laser beam was directed to every unwanted cell in the well to induce photomechanical lysis (Koller et al., 2004). This plate scanning process was repeated 1–3 times within a 1–12 day period. Following up to three sequential sub-cloning cycles on LEAP, clones were transferred to fresh 384-well plates for expansion in serum-free medium, followed by transfer to 96-well, 24-well, and ultimately 6-well plates for larger scale culture. Cells were re-analyzed with the in situ capture assay on LEAP, and were placed in suspension culture for determination of antibody secretion by ELISA. Briefly, 96-well plates (VWR; San Francisco, CA) were coated with donkey anti-human IgG (Jackson Immuno Labs; West Grove, PA). Following overnight incubation at 4°C, wells were blocked with BSA. Extensive washing steps were performed to minimize background. Unknown samples at three different dilutions, and positive and negative controls, were added to the plate and incubated for 2 h at 4°C. Eight dilutions of human IgG stock solution (Jackson Immuno Labs) were used to construct the standard curve. Horseradish peroxidase (HRP)-labeled donkey anti-human IgG F(ab')<sub>2</sub> fragment (Jackson Immuno Labs) was added at 1:25,000 dilution, followed by another series of washes. The

substrate for HRP was 3,3',5,5' tetramethylbenzidine (TMB; Pierce; Rockford, IL) used at 50  $\mu$ L per well. Following acid addition, color was measured at 450 nm using an absorbance plate reader (Tecan; Durham, NC).

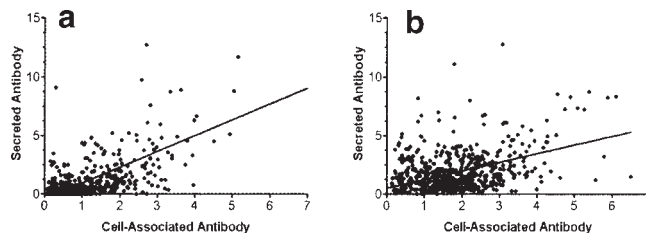
## RESULTS AND DISCUSSION

### In Situ Measurement of Specific Antibody Secretion

Development of the LEAP<sup>TM</sup> system which combines high-throughput in situ cell imaging with laser-mediated cell purification was recently reported, demonstrating high-efficiency cell purification and cloning based on cell-associated markers (Koller et al., 2004). To enable in situ measurement of specific antibody secretion from individual cells on LEAP, a novel capture and detection method was developed. Recombinant CHO cells producing humanized antibody were plated in serum-free medium into 96-well plates that had been coated with a capture matrix containing protein G. After 6–48 h of incubation, followed by blocking and washing steps, wells were stained with CellTracker Green and PE-conjugated goat anti-human IgG antibody fragment. Wells were washed and plates were then placed in the LEAP instrument for multi-color imaging and laser processing (Koller et al., 2004). Software algorithms were developed to locate each cell in the green image creating a kernel, and then grow each kernel into the area surrounding each cell until adjacent kernels were encountered (Fig. 1a). Quantification of red staining in the area of each cell (i.e., green area) and the kernel area around each cell (but excluding the cell area) allowed calculation of cell-associated and secreted antibody on an individual cell basis, respectively (Fig. 2a). Note the great degree of heterogeneity of specific antibody secretion within this cell line. Approximately 15% of the cells showed very little antibody present



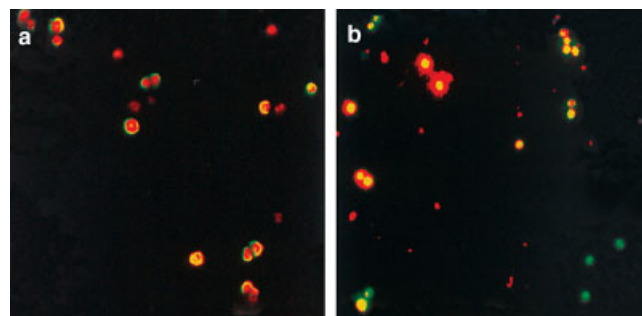
**Figure 1.** In situ capture, detection, and quantification of individual cell antibody secretion. Recombinant CHO cells were cultured on a capture matrix resulting in localization of secreted antibody in the vicinity of each cell. Cells were stained with CellTracker Green, and the recombinant antibody was stained with a PE-conjugated anti-human IgG antibody fragment. **a:** Photomicrograph of the parental cell line, showing that the amount of secreted antibody (red) around each cell (green) varied widely. Software algorithms automatically determined the location of each cell and created a region around each cell (white box) for determination of secreted antibody to be associated with each cell. **b:** Photomicrograph of the derived clone after 50 population doublings, showing more homogeneous and stable antibody secretion (red) from each cell (green) in the population.



**Figure 2.** Scatter plot of secreted and cell-associated antibody. Distribution of values for **(a)** the parental population represented in Figure 1a ( $r^2 = 0.34$ ;  $n = 909$ ), and **(b)** the derived clone population represented in Figure 1b ( $r^2 = 0.06$ ;  $n = 646$ ).

on or around the cell. Another 30%–40% of the cells stained for antibody on the cell surface but did not appear to result in much antibody secretion. Most of the secreted and captured antibody was associated with about 30% of the cells within the well. The relative amount of captured antibody detected around each cell spanned up to a 10,000-fold range indicating great heterogeneity within the parental cell population.

There have been conflicting reports in the literature regarding the correlation between the antibody detected on (and in) cells and the amount of antibody secreted from a cell population (Brezinsky et al., 2003; Cherlet et al., 1995; Gray et al., 1995; Meilhoc et al., 1989). The method developed here allowed direct examination of the cell-associated and secreted antibody from a large number of individual cells. The resulting data demonstrate a clear lack of correlation between cell-associated and secreted antibody, with correlation coefficients ranging from 0.10 to 0.35. The above approach with CHO cells was further generalized and verified with recombinant NS0 cells and hybridomas (Fig. 3), collectively representing the most widely used cell lines in commercial therapeutic protein production (Walsh, 2003). Significant heterogeneity of antibody secretion on an individual cell basis and lack of correlation between cell-associated and secreted antibody was observed for all cell types, demonstrating the general nature of these findings.



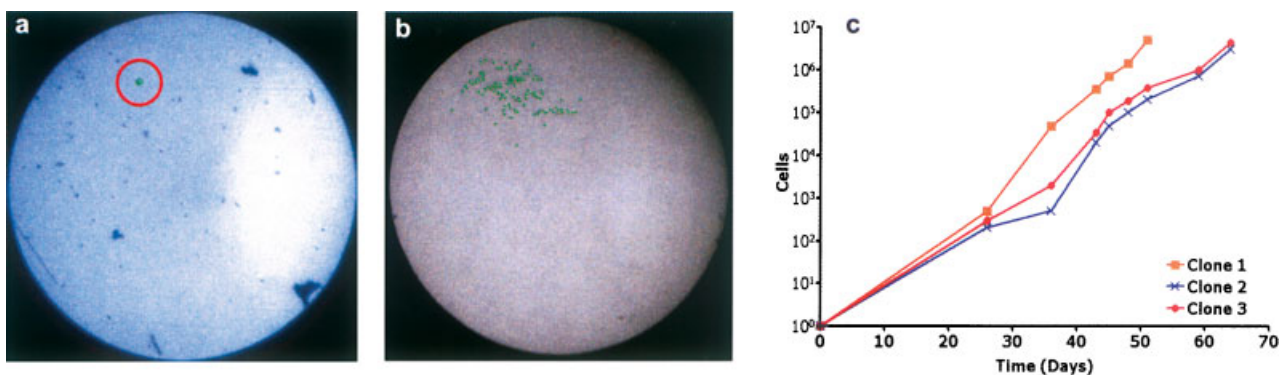
**Figure 3.** In situ detection of cell secreted antibody in a variety of cell lines. Cells were stained with CellTracker stain ((a) orange or (b) green), and their secreted antibody was captured using Protein G. Detection was performed using a (a) FITC- or (b) PE-conjugated anti-human IgG F(ab')<sub>2</sub> antibody. Images were captured using FITC and PE filter sets, overlaid and pseudo-colored. Shown are (a) NS0 cells and (b) murine hybridomas.

## Laser-Mediated In Situ Cell Cloning

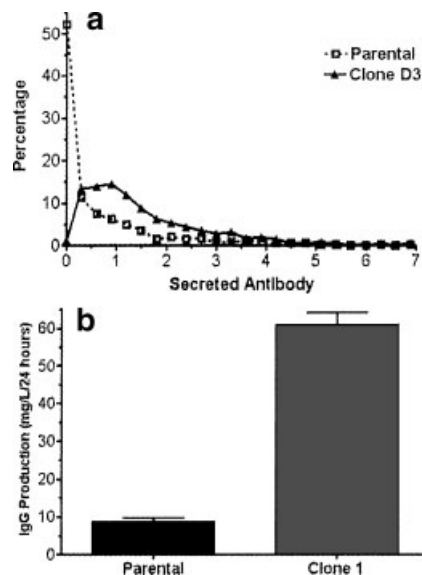
Because a minority of cells appeared to be responsible for the majority of secreted antibody, a cloning strategy was developed to select the highest-secreting cell within each well using in situ laser-mediated cell purification on LEAP. Software algorithms were developed and used to automatically locate and rank all cells in a well based on the amount of secreted antibody around each cell and several other parameters (e.g., cell area, kernel area, proximity to other cells, etc.). The highest-ranking cell in each well was spared while all other cells in the well were automatically targeted and eliminated by laser-mediated photomechanical cell lysis (Koller et al., 2004). This entire process (i.e., imaging, ranking, and shooting) occurred automatically in real time, requiring <30 s to process each well containing up to 10,000 cells. Elimination of all but one viable cell per well was verified by viability staining and imaging of wells (Fig. 4a). In some cases, an island of helper cells around the clone of interest was temporarily spared from elimination to improve local medium conditioning and cloning efficiency, particularly when using serum-free medium for cloning. After several days, such wells were re-processed on LEAP to eliminate the helper cells, resulting in a verified and documented cloning event.

## Clone Outgrowth and Characterization

Individual cells purified in this manner were allowed to grow, resulting in clonal colonies that were transferred to larger wells for expansion. Growth of cells and colonies was automatically monitored in situ on LEAP (Fig. 4b) providing rapid determination of cell growth rates for each clone, allowing subsequent efforts to be focused on clones with acceptable growth rates (Fig. 4c). The newly derived clonal populations were expanded for >50 population doublings over several months, and were then analyzed in comparison to the parental population. Cells from the derived clone were placed into the in situ secretion assay (Fig. 1b), demonstrating a substantial increase in specific antibody secretion rate



**Figure 4.** In situ purification and clonal outgrowth of cells with high antibody secretion. **a:** Validation of a cloning event showing only one viable cell (green; calcein AM) remaining in the well following laser processing, along with some non-viable cells (blue; trypan blue). **b:** Monitoring of clone outgrowth was performed by sequential imaging of wells at various times post-processing, with automated cell counting within brightfield images (software identification of cells marked by green dots). **c:** Growth curves of parental and derived clone cells.



**Figure 5.** Evaluation of derived clone properties after fifty population doublings. **a:** Histogram of individual cell antibody secretion, demonstrating elimination of non- and poorly-secreting cells and a stable upward shift in the population secretion profile. **b:** Antibody secretion of parental and derived clone cells in static 24-h batch well plate culture as determined by ELISA. Data represent the mean of three measurements ( $\pm$ SEM).

that was remarkably more homogeneous than the parental population (Fig. 2b). A histogram of antibody secretion from the parental and derived clone clearly documents the elimination of non- and poorly-secreting cells, an upward shift in specific antibody secretion rate (3.8-fold increase in mean value;  $P < 0.001$ ), and a more homogeneous distribution of secretion ( $C_v$  decreased from 1.8 to 1.0) as a result of the in situ cloning process (Fig. 5a). Static 24-h batch well plate cultures were also used to evaluate the derived clones and parental lines, demonstrating significant 5- to 20-fold improvements in antibody secretion rate in representative experiments using different cell types (Fig. 5b).

In summary, a new in situ cloning approach has been developed to directly select cells exhibiting the greatest specific antibody secretion rate, resulting in clonal

populations with improved homogeneity and stability with respect to recombinant antibody secretion. This automated high-throughput approach has been applied to three different cell types for rapid screening of millions of cells, in each case demonstrating a consistent lack of correlation between secreted and cell-associated antibody. The unique ability to eliminate undesirable cells in a well with spatial and temporal resolution with sequential re-imaging and re-processing steps had significant benefits in this application, allowing helper cells to be initially spared and later eliminated with great precision to ensure clonality and improve cloning efficiency in serum-free medium. The ability to perform serial sub-cloning operations after only a few cell doublings significantly reduced the time necessary to arrive at a stable homogenous population to about 1 month. Cell lines with specific antibody secretion rates of >50 pg/cell per day (in static 24-h batch culture) were routinely obtained as a result of this cloning approach, often times representing up to 20% of the clones screened. With appropriate modifications of capture matrix and staining chemistries, the approach described in this report should be readily applicable to other secreted proteins, including recombinant therapeutic proteins as well as endogenous secreted proteins such as cytokines and chemokines.

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