Assessing monoclonality under screening of mammalian cell lines by ClonePix FL

Genetix

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Introduction

Isolation of candidate mammalian cell clones by limited dilution, ring cloning or simply manual collection of colonies is a time consuming, resource intensive and costly procedure that is prone to cross contamination of cells and user error. ClonePix FL is a proven highly efficient technology for automating this process, is compatible with animal free conditions and has been adopted by many of the major pharmaceutical companies. The power of ClonePix FL technology lies in its ability to visualise and quantify antibodies secreted from thousands of clones *in situ*, and to select and isolate only the highest value candidates thus bypassing the need for high throughput automation. It significantly shortens timescales, simplifies down stream culture and delivers a powerful return on investment.

ClonePix FL Technology

A fundamental initial step in the ClonePix FL technology is to culture mammalian cell lines in semi-solid media such that cells form discrete colonies, each originating from a single parent cell. Heterogeneous populations of thousands of cells are grown into clonal colonies in 6-well or 1-well plates and screened rapidly using white light to detect the colonies and by multiple fluorescent wavelengths to quantify secreted protein *in situ*. Figure 1 shows a typical distribution of clones ranked by fluorescence (secretion). The dedicated software quantifies the fluorescence associated with each colony and drives the automated picking of only the most desirable clones into 96-well destination plates. Plates of picked colonies are then grown to confluence in preparation for clone expansion and scale-up.

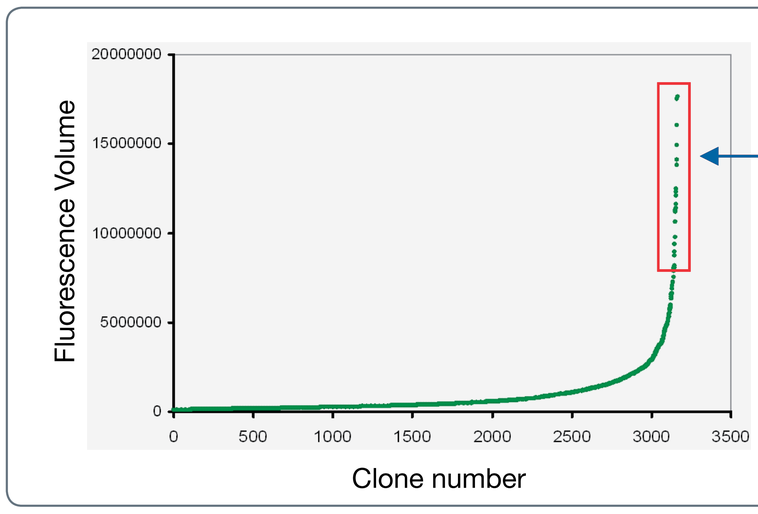

Figure 1: Representative data generated on the Genetix ClonePix FL showing fluorescence associated with each colony. Fluorescence provides a measure of a specific secreted protein. Clones with highest secretion are automatically selected for collection.

The technology has been validated for the specific detection of secreted antibodies and monomeric proteins, cell surface proteins, and intrinsic GFP fusion proteins. It is compatible with a range of cell types including hybridoma, myeloma, HEK293, and both suspension-adapted and adherent CHO cells.

Fluorescence Based Assays

The secreted protein detection assay requires the secreted product to be immobilized around the colony using a fluorescent detection probe. Figure 2 shows a cartoon representation of the detection of IgG using CloneDetect, an IgG-specific detection probe. Figure 3 shows example images generated by the ClonePix FL, identifying the highest producing clones. Multiple different proteins can be detected and isolated simultaneously by using detection agents labelled with different fluorophores (multiplexing). For hybridoma fusions, clones can be selected based on antigen-specificity by additionally adding antigen as the detection probe; this can be directly conjugated with fluorescence or via a secondary detection agent.

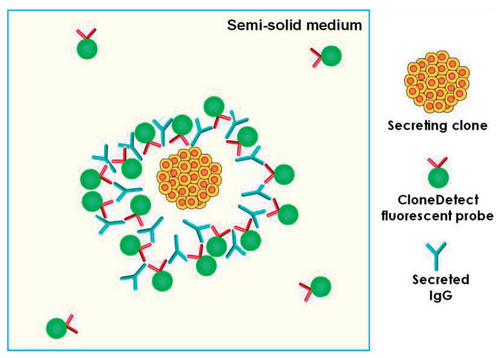

Figure 2. **Secreted protein detection assay principle.** Formation of fluorescent precipitate around a clonal colony grown in semi-solid media. Secreted IgG is cross-linked around the colony using a CloneDetect detection reagent, which quantifies the amount of secreted protein.

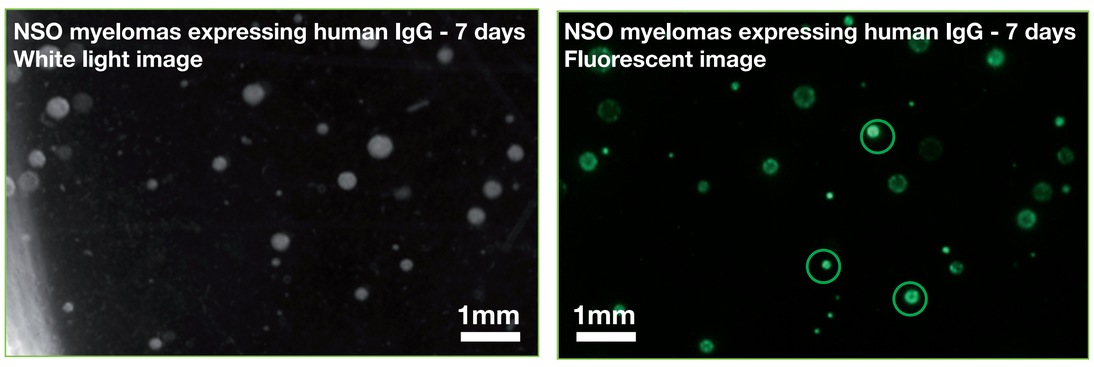

Figure 3. Fluorescent detection of a secreted IgG from an NSO myeloma cell line. An NS0 cell line expressing a human IgG against a bacterial protein was plated out and grown in CloneMatrix semi-solid media in the presence of FITC anti-IgG. After 7 days individual cells have grown into colonies (left panel), these are exposed to the in situ secreted protein detection assay to detect and quantify IgG secretion (right panel). The image is colorized and high secretors are highlighted.

Assessing Monoclonality by Statistical Evaluation

A critical aspect of the ClonePix FL technology is that the high value colonies collected should be clonal. Clonality of hybridomas collected from semi-solid medium was evaluated by Davis *et al* (1) with the conclusion that, at appropriate seeding density, the probability of coincidence (non-clonality) is 4%.

We have generated a statistical calculation that shows that there is a correlation between the % probability of monoclonality and seeding density / size of colony. The calculation is as follows:

In a typical experiment, CHO cells are plated into semi-solid medium to produce 25 colony outgrowths per 35 mm diameter well (i.e. a 6-well microplate well). At day 14, the colonies are around 0.75mm in diameter. Using the formula (1/4)*Pi*(0.75+0.75)2*(N-1) / (1/4*Pi*(35)2 where colony number n=25 the probability of coincidence is 4.4%, thus the probability of monoclonality for one round of cloning should be 95.6%. This is very close to the value proposed by Davis *et al*.

This relationship is shown graphically in figure 4.

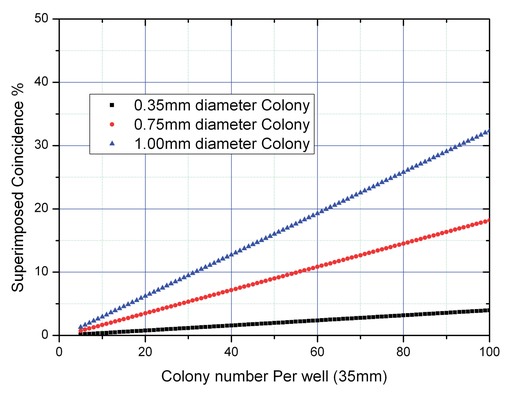

Figure 4: Graph showing the relationship between colony number / colony size and superimposed coincidence (% likelihood of two colonies growing in the same location).

Assessing Monoclonality by Experimentation

To test this experimentally, two IgG secreting hybridoma cell lines were combined. One was IgG_1 secreting and one was IgG2a secreting. Colonies were grown in CloneMatrix-based semi-solid media in the presence of fluorescently labelled isotype-specific antibodies against the IgG_1 and IgG_{2a}. AlexaFluor488-conjugated anti-IgG_1 was visualised in the FITC channel of ClonePix FL, and Alexa546-conjugated anti-IgG_{2a} was visualized in the rhodamine channel. Representative images are shown in Figure 5.

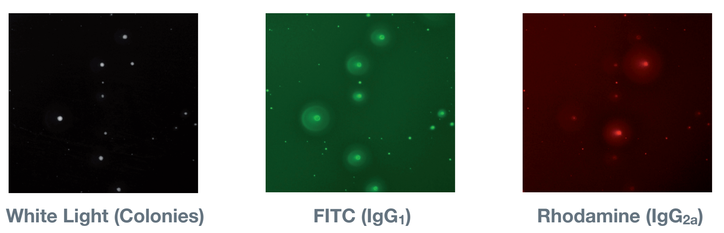

Figure 5. ClonePix FL images from the mixed hybridoma experiment. The white light image shows all colonies. The FITC image identifies colonies that are secreting IgG_1 and the rhodamine image identifies colonies secreting IgG_{2a}.

The colonies were analysed on ClonePixFL and the colonies differentially picked according to either high FITC / low Rhodamine exterior mean intensity (for IgG_1 secretors) or low FITC / high Rhodamine (for IgG_{2a} secretors). A total of 312 colonies (156 of each type were picked). The picked cells were grown in 96 well plates for 4 days, and then the conditioned media were measured for IgG_1 and IgG_{2a} using isotype-specific ELISA assays (Bethyl Laboratories). Cells with either poor outgrowth or a negative ELISA for both IgG_1 and IgG_{2a} were excluded from the results.

The results showed that of 143 colonies that were picked based on IgG_1-specific fluorescence, only one showed a low level of IgG_{2a} (clonality = 99.3%). Of 81 IgG_{2a} colonies picked based on IgG2a-specific fluorescence, three were heterogeneous (clonality = 96.4%). Monoclonality for the complete data set was 98.25%. The results are summarized in Figure 6.

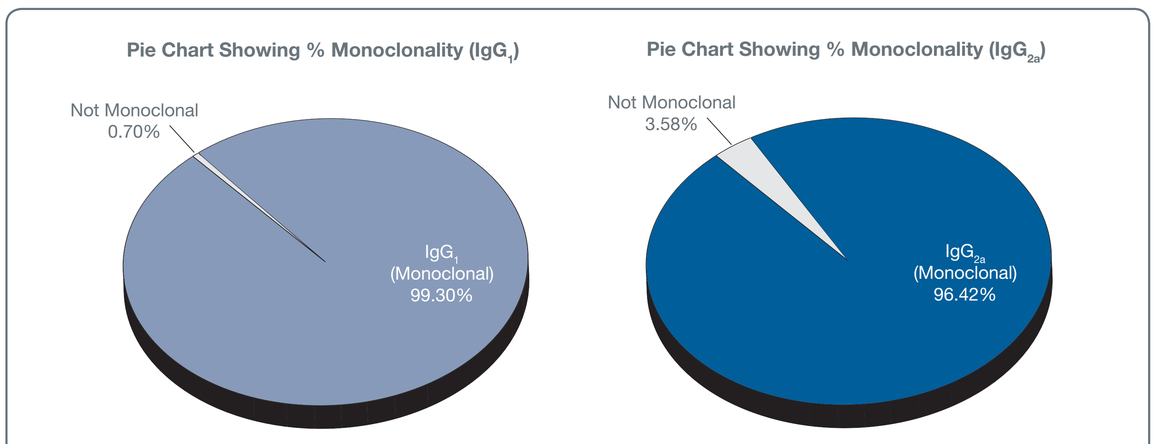

Figure 6. Pie charts showing clonality of picked clones.

These experimental data validate the statistical evaluation that clonality after a single round of picking on ClonePix FL is >95.6%. Based on the statistical evaluation, it can be further predicted that by performing a second round of cloning (re-plating the picked clones and re-picking on ClonePix FL) the probability of coincidence would be 4.4%*4.4%=0.2%, a probability of monoclonality of 99.8%. Picking at an earlier timepoint (for example when colonies are only 0.35mm in diameter), the probability of monoclonality can be increased further to 1-0.96%*0.96%, or 99.99%.

Summary and conclusions

ClonePix FL has been demonstrated to be a powerful tool for rapid screening and isolation of high value secreting mammalian cell lines, for use in the production of therapeutic proteins and research monoclonal antibodies. The data presented here supports the original observations of Davis *et al* that picking colonies of mammalian cells from semi-solid medium is an effective means to isolate clonal candidate cell lines.

References

1. Davis, J.M., Pennington, J.E., Kubler, A.M. & Conscience, J.F. A simple, single-step technique for selecting and cloning hybridomas for the production of monoclonal antibodies. J. Immunol. Methods 50, 161–171 (1982).

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