Rapid selective *in situ* screening of hybridomas in semi-solid medium

Anan Chuntharapai*, Christopher J. Mann†, Steven Watters† and Julian F. Burke†

* Genentech, 1 DNA Way, South San Francisco, CA 94080-4990, USA † Genetix, Queensway, New Milton, Hampshire, BH25 5NN, UK

Introduction

- Mammalian cell culture technology has been thrust to the forefront of the biopharmaceutical industry because of the superior fidelity of animal cells in producing clinically relevant proteins
- Costs for developing new therapeutic proteins are high and the industry is under pressure to shorten time to market
- A major bottleneck in both discovery and development of cell lines is screening large heterogeneous populations of cells to find the best cell line candidates
- ClonePix FL technology has previously been shown to find and isolate high producing candidate clones for production in just 1-2 weeks[1]
- Here we present a simple one-step hybridoma selective secretion assay for detecting and isolating antigen-specific, IgG-secreting hybridoma clones in as little as 8 days from fusion with minimal labor

Methods

Hybridoma generation

- Mice were immunized against pharmaceutically relevant antigens tagged with human Fc (range 52-160kD) and hybridoma fusions generated

Selective secretion assay

- After overnight recovery, part of a fusion was mixed gently with the following components
  - Semi-solid medium containing 10% FBS
  - HAT (Hypoxanthine, Aminopterin, Thymidine)
  - Goat anti-mouse IgG (5µg/mL)
  - Antigen tagged with human Fc (5µg/mL)
  - FITC-conjugated Goat F(ab')$_2$ anti-human Fc (1.3µg/mL)
- The mixture (50mL final vol.) was plated into up to ten 6-well plates at 2mL per well
- Plates were incubated at 37°C, 5% CO_2 for 7 days to grow discrete hybridoma colonies
- Using ClonePix FL, plates were imaged by FITC, and positive clones picked (5µL vol.) into 96-well plates pre-filled with liquid medium (150µL per well)

Antigen-specific capture ELISA

- The picked clones were expanded for 3 days, and then medium was replaced to measure 24-hour productivity
- ELISA plates were coated with Fc specific goat anti-human IgG (2.0µg/ml), incubated with human Fc-tagged antigen (0.4µg/ml), incubated with the conditioned media samples, and then probed with HRP sheep anti-mouse IgG and TMB
- The ELISA data were corrected for cell density using CloneSelect Imager (Genetix)

Principle of the hybridoma selective secretion assay

Concomitant with hybridoma colony growth and HAT selection in semi-solid medium, the anti-mouse IgG diffuses through the medium and creates a local precipitation complex in the vicinity of IgG-secreting hybrids. Non-secreting hybrids or those secreting other immunoglobulin types will not form a capture complex. Only those IgG hybrids that secrete antigen-specific IgG will accumulate fluorescence via the Fc-tagged antigen. The principle is illustrated in **Fig 1**.

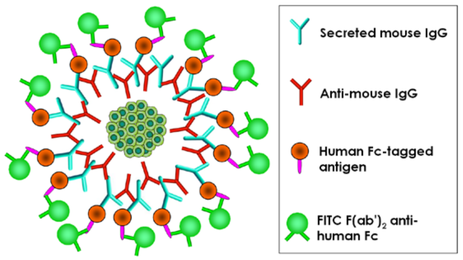

Figure 1. Principle of the hybridoma selective secretion assay.

The ClonePix FL software automatically detects all colonies using white light imaging, and the antigen-specific IgG-secreting colonies using fluorescence (up to 5 different wavelengths). Non-clonality can be avoided by excluding colonies in close proximity, and unfused antigen-specific IgG-secreting B-cells can be excluded by their much smaller size.

Results and Discussion

Selective picking

To investigate the effectiveness of the selective secretion assay, a hybridoma fusion was generated from a mouse immunized with a 160kD antigen, and then part was assayed as in Methods. All hybrid clones identified as FITC positive by ClonePix FL were picked first (n=179, **Fig2A**). Then all remaining discrete clones were picked using white light (n=346, **Fig2B**). In a parallel control experiment, no colony-associated fluorescence was observed using mouse-Fc-tagged antigen, indicating that clone-specific fluorescence was mediated through the human-Fc tag.

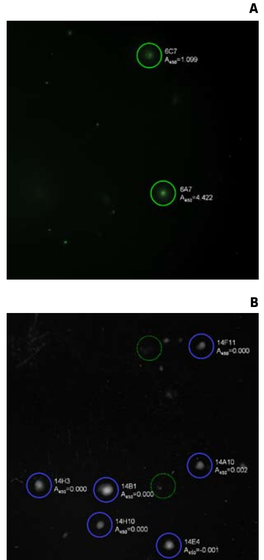

Figure 2. Detection and picking of hybrid colonies from semi-solid medium using FITC imaging for antigen-specific clones (A, green circles) and then white light imaging for non-specific clones (B, blue circles). Exposure times per image: FITC 5s; white light 1s.

Growth of clones after picking

The growth of clones after picking is shown in **Table 1**. 78% of the FITC positives and 99% of those picked subsequently using white light showed good outgrowth after collection. The high viability of the positive clones compares favourably with that observed using cell sorting or limiting dilution techniques.

	Total picked	Clones with good growth	% clones with good growth
Picked by FITC	179	139	78%
Picked by white light	346	343	99%

Table 1. Growth of clones after picking, measured using CloneSelect Imager.

Confirmation by ELISA

To test if the secretion assay selectively found the right clones, a capture ELISA was established to measure the presence of antigen-specific IgG in the harvested supernatants (**Fig 3**).

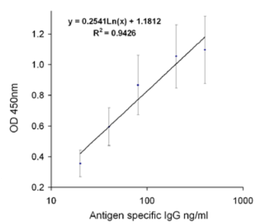

Figure 3. Dilution curve for antigen-specific IgG capture ELISA. One curve was set up per 96-well plate. Values are mean ±SD for all dilution curves used in the study.

The ELISA results presented in **Table 2** and **Fig. 4** show that the FITC-positive clones had a mean ELISA measurement 122-fold higher than those picked subsequently by white light (P<0.0001, two-tailed unpaired t-test). Assuming an OD_{450} cut-off of 0.5, 89% of fluorescent clones were antigen positive by ELISA. Importantly, only one clone or 0.3% of the white light group returned a positive result by ELISA, showing that the secretion assay is a powerful tool for selecting antigen-positive IgG-secreting hybrid clones.

	ELISA (OD_{450} normalized)	ELISA positive ($OD_{450} \geq 0.5$)	ELISA negative ($OD_{450} < 0.5$)
Picked by FITC (n=139)	2.197±2.363	124 (89.2%)	15 (10.8%)
Picked by white light (n=343)	0.018±0.061	1 (0.3%)	343 (99.7%)

Table 2. Distribution of picked clones by antigen-specific ELISA. Only clones with good growth after picking were included.

The observation that 11% of fluorescing clones were reported by ELISA to be antigen negative might be due to hybrid instability, since this was measured 4 days after clone picking. An alternative explanation is that they were low affinity positives that were undetectable by ELISA, which requires multiple washing steps. By selecting clones as early as 8 days after fusion, it is likely that some surviving unfused B-cells will be collected, or even dead B-cells that created fluorescent precipitate before death. Although most can be excluded from selection based on their small colony size, the collection of some false positives is not critical as these will not survive under continued HAT selection. The results indicate that this method is an effective means for screening antigen positive clones *in situ*, and for eliminating the need to process antigen-negative or non-IgG clones.

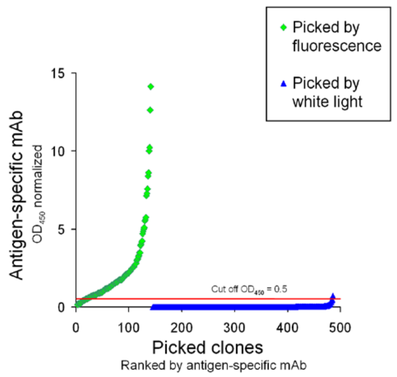

Figure 4. Measurement of antigen-specific IgG by ELISA of clones picked by fluorescence (green) and subsequently by white light (blue). Data shows only those clones with good growth after picking.

We next questioned whether the antigen-specific fluorescence associated with each clone might provide a measure of hybridoma IgG secretion rate. Probing human IgG-transfected CHO-S cell populations with fluorescently-conjugated anti-IgG has previously shown good correlation between IgG-specific fluorescence and IgG productivity.[2] However, no correlation was observed between fluorescence measured in the secretion assay and subsequent ELISA measurements (R2=0.0444, NS). The reasons for this discrepancy are unclear. If an estimate of the secretion rate of antigen-positive clones is required, a dual fluorescence approach may be preferable where fluorescently-conjugated anti-mouse IgG could quantify secretion from the antigen-positive clones.

The described assay was used to screen a further 8 antigens with the same human-Fc tag. In each case, part of the hybridoma fusion was screened using the selective secretion assay and part was randomly screened. **Table 3** shows the number of colonies picked and the desired candidates found following additional screening to identify site-specific clones (and to eliminate those secreting antibody against Fc-tag). Across the panel of antigens, the selective secretion assay collected only 27% of the number of clones collected by random screening, but contained 70% more candidates. Hence, for the same number of clones collected and processed, the selective secretion assay found an average of 6.3 times more candidate clones. In one experiment (antigen E), collection of 1739 random clones was unable to identify any candidates, whereas selective collection of only 98 clones was able to discover a candidate clone. This indicates that the assay's greatest resolving power is in finding rare clones specific for weakly immunogenic proteins.

	Selective secretion assay		Random screen	
Antigen (kD)	Colonies picked	Candidates found	Colonies picked	Candidates found
A (160)	64	3	144	3
B (160)	424	7	1440	4
C (160)	64	2	532	2
D (160)	583	3	864	2
E (110)	98	1	1739	0
F (75)	30	6	304	6
G (52)	259	13	975	2
H (100)	745	4	2304	4
Total:	2267	39	8302	23

Table 3. Comparison of the selective secretion assay and random clone selection for 8 hybridoma fusions.

Conclusions

- Hybridoma clonal cell lines that are IgG-secreting and antigen-specific can be selectively screened and isolated in a rapid one-step automated process
- The time taken from fusion to near-confluency at the 96-well stage takes as little as 11 days and requires only 5 hours of labor per fusion
- The method is highly efficient and requires minimal cell handling which leads to high cell viability
- The assay greatly reduces downstream processing permitting more fusions to be screened
- The use of Fc-tagged antigen as detection probe avoids the need to chemically conjugate the antigen and provides a universal antigen detection method
- The described ClonePix FL assay is compatible with other methods that shorten the hybridoma discovery process such as RIMMS (Repetitive Immunization Multiple Sites) that together can generate high value cell lines in as little as 2-4 weeks [3,4]

References

1. Mann, CJ *et al*. Automated screening and selection of antibody-producing mammalian cell lines. Lab Automation meeting. San Jose, Jan 2005.
2. Watters, S *et al*. High throughput screening and selection of stable high secreting clones. ESACT UK meeting. Wyboston, Jan 2007.
3. Bynum, J *et al*. Development of class-switched, affinity-matured monoclonal antibodies following a 7-day immunization schedule. Hybridoma 18, 407-411 (1999).
4. Lane, JR *et al*. Antibodies that identify only the active conformation of Gi family G-protein α subunits. FASEB J.22 (6), (June 2008).

Genetix

www.genetix.com

0308-17