Screening and isolation of antigen specific IgG–secreting hybridoma clones in a rapid one–step process

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Introduction

- The pharmaceutical industry is under pressure to reduce development time for new therapeutic proteins in a cost-effective manner
- A key rate-limiting step is screening large populations of cells for best cell line candidates
- We have previously reported a technology for obtaining high-value clones in just 1-2 weeks[a]
- We report here the targeted isolation of antigen specific IgG-secreting clones from a hybridoma fusion in a rapid one-step process

Methods

- A mouse was immunized against soluble antigen (160kD) and a hybridoma fusion generated
- After overnight recovery, part of the fusion was mixed gently with the following components
  - Methylcellulose-based semi-solid medium containing 10% FBS and HAT
  - 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 four 6-well plates at 2mL per well
- The plates were incubated at 37°C, 5% CO_2 for 7 days to grow discrete hybridoma colonies
- The plates were imaged by fluorescence and FITC positive clones picked using ClonePix FL
- The plates were then imaged by white light and the remaining non-fluorescent clones picked
- The selected clones were collected (5µL vol.) into 96-well plates pre-filled with liquid medium (150µL per well)
- Picked clones were incubated at 37°C, 5% CO_2 for 3 days
- The medium was removed and replaced with fresh medium (200µL), and the plates incubated for a further 24 hours
- The confluence of all wells was recorded, and then the conditioned medium was harvested and tested for antigen specificity by ELISA
- A small number of cells from selected high fluorescing and non-fluorescing clones were re-plated to semi-solid medium (2 x 2mL) to assess the secretion from daughter clones

Results

Selective picking

All hybrid clones identified as FITC positive by ClonePix FL were picked first (n=179, Fig1A). All remaining discrete clones were then picked using white light (n=346, Fig1B).

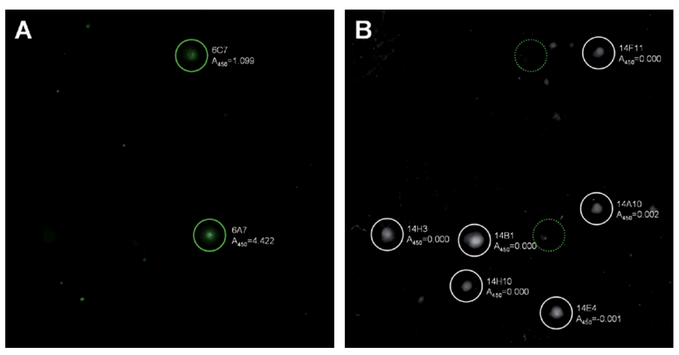

Figure 1. Picking of hybrid colonies from semi-solid medium using ClonePix FL by FITC imaging to collect antigen-specific clones (A) and then by white light to collect non-specific clones (B). Exposure: 5 sec for FITC; 1 sec for white light.

Growth of picked clones

The confluence of all clones was measured 4 days after picking (Fig 2 and Table 1). Nearly every pick successfully collected a colony, although confluence of those picked by fluorescence was lower (49±35%) than for those picked by white light (70±21%).

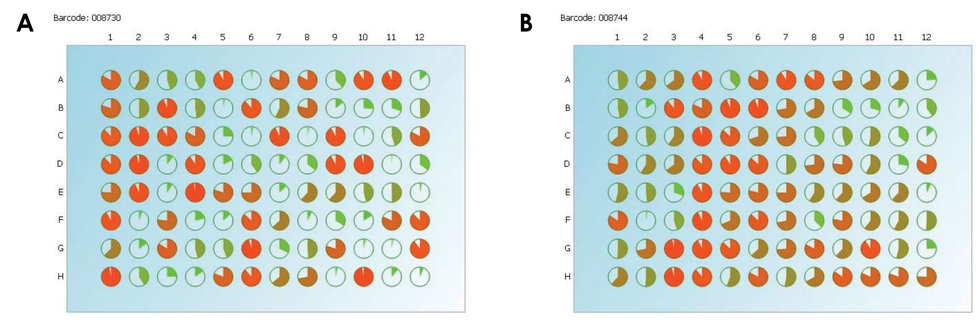

Figure 2. Confluence measurements shown as pie charts for each picked clone obtained using CloneSelect Imager. One representative plate is shown for FITC-positive clones (A) and one for clones collected by white light (B).

	Total picked	>1% confluence	>5% confluence	>20% confluence
Picked by fluorescence	179	178 (99.4%)	157 (87.7%)	123 (68.7%)
Picked by white light	346	345 (99.7%)	344 (99.4%)	335 (96.8%)

Table 1: Confluence of all clones was measured 4 days after picking

Antigen specific capture ELISA

An ELISA was established to measure the presence of antigen-specific IgG in harvested supernatants. The average of all standard curves used in the study is shown in Figure 3.

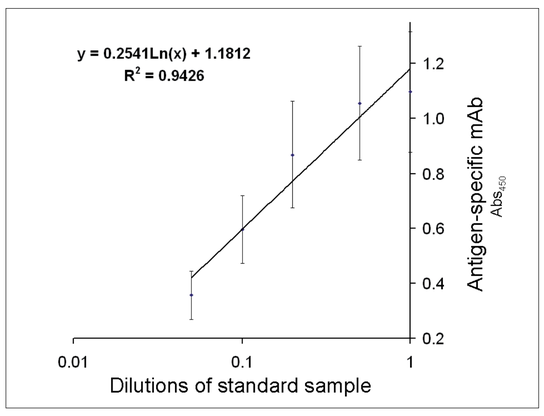

Figure 3. Average of all standard curves used in the study. Briefly, plates were coated with 2.0 µg/ml goat anti-human IgG (Fc specific), blocked, and then incubated with 0.4 µg/ml human Fc-tagged antigen. Cultured supernatants and dilutions of standard sample (100µL) were incubated for 1 hour, and then the plates probed with sheep anti-mouse IgG-HRP.

Antigen specificity results

The aim of this study was to find antigen specific positive clones rather than highest secretors, so all data are shown as ELISA absorbance values corrected for background (Abs450 - Bk). The mean ELISA value for the fluorescent picked clones with confluence >1% was 0.938±0.779, and was dramatically higher than for the subsequently picked white light population (0.009±0.030). The highest 10 values for each population are shown in Table 2.

Picked by fluorescence		Picked by white light	
Plate/Well	A450-Bk	Plate/Well	A450-Bk
6H8	5.764	16F2	0.524
6A7	4.422	14G12	0.082
6H10	4.422	15D2	0.079
6C12	2.701	15B6	0.077
6F11	2.642	17G7	0.071
6G9	2.448	17B8	0.053
6E12	2.188	14C5	0.052
6D7	2.167	17E7	0.049
6E9	2.119	17F7	0.041
6B12	2.119	16B1	0.038

Table 2. The top 10 antigen specific clones in each group. Positives are shown in red. An antigen ELISA background-corrected value of Abs_{450} <0.2 was considered negative.

Assuming a cut-off Abs_{450} – Bk value of >0.2, 90% of clones picked by fluorescence were antigen positive by ELISA (Table 3). One explanation why 9.6% were found to be antigen negative is that low affinity positives found by ClonePix FL may be undetectable by ELISA, which requires multiple washing steps. Only one clone measured as antigen positive by ELISA was collected in the white light population.

	Antigen positive	Antigen negative
Picked by fluorescence	161 (90.4%)	17 (9.6%)
Picked by white light	1 (0.3%)	344 (99.7%)

Table 3. Distribution of antigen positives and negatives between the two populations. An antigen ELISA background-corrected value of Abs_{450} <0.2 was considered negative.

Figure 4A shows the data from Table 3 graphically. We questioned whether the different levels of confluence might influence the results. Figure 4B shows that normalising confluence to 100% did not alter the pattern of distribution.

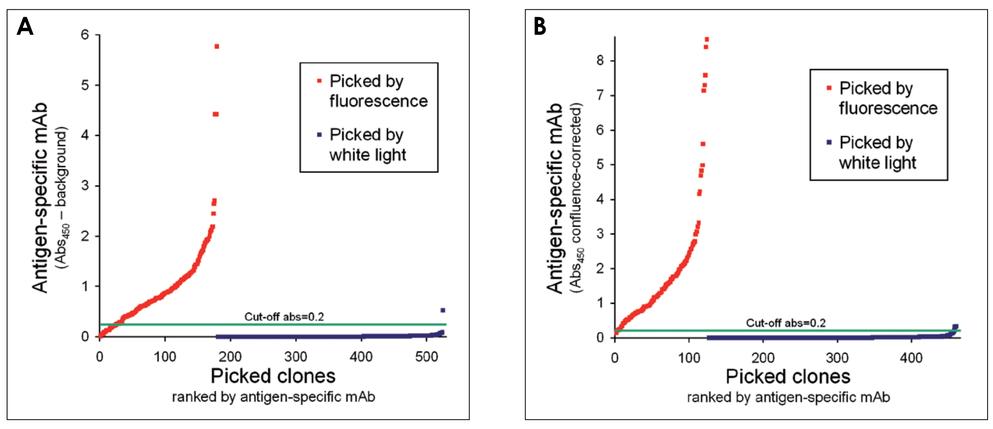

Figure 4. Selective picking by fluorescent detection of secretion is a powerful way to collect only the antigen-specific hybrids. Results were comparable when plotting all clones >1% confluence without correction for cell density (A) and when plotting only clones with >20% confluence normalised to 100% confluence (B).

Interestingly, despite fluorescent detection *in situ* being a powerful tool for detecting antigen specificity, there was no correlation between antigen-specific fluorescence and antigen-specific mAb measured by ELISA (R^2=0.0444). One possible explanation is that ClonePix FL measures antigen binding in solution phase, while ELISA measures in solid phase. The latter is known to cause antigen conformational change. Previous work has shown that there is a good correlation between IgG-specific fluorescence measured by ClonePix FL and IgG productivity[b]. Therefore, a dual fluorescence approach may be necessary where selection of highest secreting antigen positives is required.

Re-plating of clones

An aliquot of cells from fluorescing and non-fluorescing clones were re-plated to semi-solid medium to visually confirm the antigen-specificity of the sub-clones. Figure 5 shows that positively selected clones produce antigen positive sub-clones (A, B), while negative clones do not (C, D). Some fluorescing clones show variability of secretion from the sub-clones, indicating poor stability (E, F).

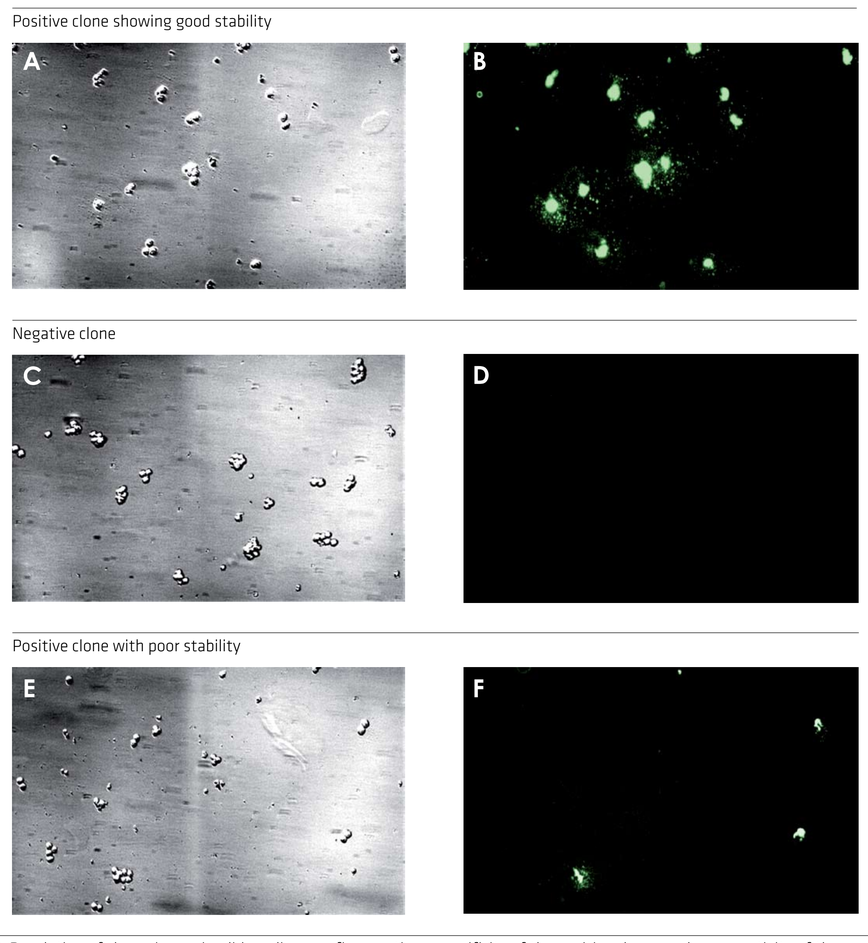

Figure 5. Re-plating of clones in semi-solid medium confirms antigen-specificity of the positive clones and non-reactivity of the negative clones. Four days after picking, one quarter of the cells of each clone were re-plated into CloneMatrix semi-solid medium with components as described in Methods except that FITC-conjugated antigen was used. The images were captured after 2 days incubation using a prototype CellReporter (Genetix). Left panels show white light, right panels show FITC fluorescence (colourised). All exposure times 90ms.

Conclusions

1) Antigen specific hybridoma clones can be screened and selectively isolated in a rapid one-step automated process
2) The process bypasses the need for traditional labour intensive high throughput screening methods, and thus permits much larger populations to be screened
3) The trapping of only secreted IgG in the vicinity of the hybrid colonies ensures that the selected clones are IgG secretors
4) ClonePix FL may provide a superior comparative measure of antigen specificity than solid-phase based ELISA
5) The antigen probe can be directly-conjugated with a fluorophore (Fig 6) or can be chased with fluorescent anti-tag antibody (Fig 1). The latter eliminates the need for chemical conjugation
6) The time taken from plating out the fusion to harvesting the media from clones for ELISA was 11 days, and required only 5 hours of labour.

References

a) Mann, CJ *et al.* Automated screening and selection of antibody-producing mammalian cell lines. Lab Automation meeting. San Jose, Jan 2005.
b) Watters, S *et al.* High throughput screening and selection of stable high secreting clones. ESACT UK meeting. Wyboston, Jan 2007.