Antigen specific antibody-producing clones selected by isotypes using the CellCelector™ system

BioSystems International (BSI) discovers novel biomarkers by performing monoclonal antibody-proteomics profiling for specific diseases. Recently, we have reported the discovery of lung cancer associated biomarkers with potential for clinical applications based on the technology. We have screened the biomarkers in a sandwich assay using monoclonal antibodies (mAbs) directed against two non-overlapping epitopes. These mAbs including an anti-leucine-rich alpha-2-glycoprotein-1 (anti-LRG1), capable to measure the differential protein level in plasma, were generated and isolated from an internal large hybridoma collection.

This application note will focus on a rapid method for isolating antigen specific clones by introducing isotype specific labeling of the heavy chain of the hybridoma secreted antibody during the cloning process using the CellCelector™ system.

The CellCelector™ system

The automated isolation of antigen specific hybridoma clones was performed using a robotic cell selection and isolation system (CellCelector™, ALS GmbH). The harvesting process is supported by an inverted microscope that offers cell observation in bright field, phase contrast and fluorescence illumination as well. The CellCelector's working process can be divided in three independent steps: Imaging, harvesting and documentation (figure 1).

The entire process from imaging to harvesting is documented via real-time images and a corresponding particle result list, which contains all predefined settings and filing positions. The CellCelector™ software translated result lists into user-defined graphics and allows exporting numeric data in standard analysis packages.

Image detection

The imaging system scanned the wells of the culture dish and automatically generated an overview image of the detected cell clones within the selected well. User-defined gray-value or colour thresholds were used to detect the target cell clones. Additional detection parameters, like diameter and shape factor were adapted to identify target cell clones and to exclude unwanted particles from the detection process. The results are recorded in the particle map and the corresponding interactive particle list is automatically stored in the data base for future analysis and subsequent automated harvesting.

Automated Harvesting

The picking list was created by selecting the clones based on the presence of fluorescent halo corresponding to mAb precipitation. Aspiration was performed using tips with a diameter of 800 µm. The following picking parameters were used: 25 µL aspiration volume, aspiration velocity 85 %, with scrape tool. Images of the colony before and after the pick were recorded automatically.

Figure 1 | Assembly of ALS' CellCelector™ : **(a)** Inverted microscope **(b)** Robotic arm **(c)** Decktray for tips and destination plates **(d)** Motorized stage

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Sandwich assay using mAbs secreted by hybridomas

Performing a sandwich ELISA as shown on **figure 2** requires two paired antibodies that bind on different epitopes of the protein (in this case LRG1). In order to screen cell culture supernatants for the optimal paired antibodies using a generic anti-mouse antibody labeled for detection, two different isotypes are needed.

We have screened the supernatants of hybridomas generated from fusion of splenocytes of mice immunized with a complex mixture of plasmatic proteins with a strong indication of antibodies recognizing LRG1 (1).

500 Hybridoma supernatants from this fusion were screened for anti-LRG1 specific antibodies by ELISA using cytochrome c as antigenic capture (2). The antibody isotypes were analyzed in parallel using appropriate Ig subclass-specific antibodies (IgG1, IgG2a, IgG2b, IgG3, IgM, from SouthernBiotech).

The wells positive for LRG1 showed the presence of different isotypes in most of the cases, suggesting the need for cloning the antibody producing cell population with the desired specificity. We have performed the cloning in methylcellulose (MC) since this method has multiple advantages as compared to the standard cloning by limited dilution and has the potential to combine the cloning process with the separation of the IgG cell population by specific isotype in one step.

Isolation of anti-LRG1 producers in methylcellulose medium with the CellCelector technology

0.9mL of cell suspension was mixed with 9mL of MC medium containing an in-house mixture of FITC conjugated anti-mouse IgG1 plus TRITC conjugated anti-mouse IgG2a/IgG2b (**figure 3**), and platted in 8cm diameter Petri dishes. The cell count of the cell suspension was adjusted for optimal platting density. The growth efficiency in the MC media was around 30% and the distance between colonies was more than 1.2mm to insure optimal picking with the 800µm capillary module. After 4 days of incubation, using the CellCelector system, the plates were first scanned in bright field to determine the total number of colonies and then processed automatically in dual color fluorescence, to identify the isotype of each antibody secreting colony (**figure 4**). Results were compiled in a table using ALS implemented application software and analyzed for selective picking of labeled clones. The picking was performed by an automatically driven robotic arm with the parameters described previously and the colonies were transferred to a 96-well tissue culture plates.

Sandwich partners for LRG1 detected by isotyping

Using the method described above, 8 cloning experiments provided 8 confirmed cell lines with three different isotypes, allowing the establishment of an appropriate sandwich paired antibodies (**Table 1**). The results indicate that a single IgG producing clone which is present only at 1 or 5% in the total cell population can be cloned by eliminating the non-IgG and IgM producers (ID269 and ID1234). Cloning of cell lines where no IgM producing cells are present (ID108, ID1095, ID238) is easily achieved in a single step process. To further characterize the antigen specificity of the clonal cell lines, a maximum of 20 sub-clones per hybridoma were selected and picked. The selection of picking was essentially based on the isotype specific labeling and all isotypes measured in the initial mix were harvested. Indeed, half of the initial hybridoma mixes presented at least two different isotypes before cloning and at this point we ignored which clonal population had anti-LRG1 affinity. Selected clones were cultured in liquid medium and supernatants were screened with LRG1 via the cytochrome c assay to identify the specific candidates. We calculated clone efficiencies (number of clones producing anti-LRG1 divided by picked IgG producers) first with all the clones picked and then in splitting the ratio by isotypes (IgG1 specific LRG1 and IgG2a/2b specific LRG1). For three hybridomas (ID108, ID238 and ID460) where the global clone efficiency was low, 8%, 33% and 13% respectively, isotyping of the colonies during the cloning process allowed us to retrieve a monoclonal antibody against LRG1 protein (table 1). Surprisingly, LRG1 specific antibody producing cell populations are clearly distinguished by the isotype in the initial hybridoma mix, suggesting an initial oligoclonal state of few independent cell lines.

The selected candidates contained two monoclonal antibodies with different isotypes and binding different epitopes of LRG1, thus allowing us to establish a sandwich ELISA for this protein.

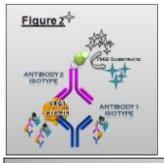
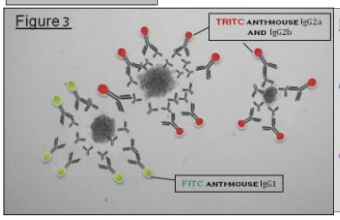


Figure 2 | Scheme of a sandwich assay for LRG1 protein using a specific capture antibody (Antibody 1) and a non-overlapping epitope antibody for detection (Antibody 2) with a different isotype.



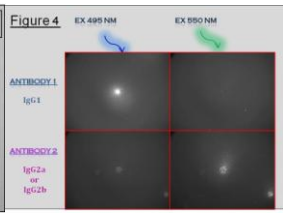


Figure 3 | A composite figure where an image of colonies growing in methylcellulose media in bright field, was superimposed with a scheme showing IgG secreting antibodies detected by fluorochrome conjugated antibodies (FITC and TRITC) to distinguish the isotype of each antibody producing clone.

Figure 4 | Images of colonies at two excitation wavelengths showing fluorescence from the precipitated antibodies secreted from the colony
The antibodies were revealed by FITC anti-mouse IgG1 for Antibody 1 and TRITC anti-mouse IgG2a/2b for Antibody 2.

	CELL POPULATION				PICKING		CLONE EFFICIENCY		
Hybridoma ID	Hybridoma Isotypes	Nber of colony	Nber of fluorescent colony	% IgG producer (a)	Nber of colony picked FITC	Nber of colony picked TRITC	LRG1 clone candidate Isotype	% Global clone efficiency (b)	% Isotype clone efficiency (c)
ID103	IgG2a	24	8	33%	0	5	IgG2a	100%	100%
ID108	IgG2a/IgG1	63	63	100%	11	1	IgG2a	8%	100%
ID1095	IgG2b	39	39	100%	0	13	IgG2b	100%	100%
ID1234	IgG1/IgM	63	3	5%	1	0	IgG1	100%	100%
ID238	IgG2a/IgG1	106	106	100%	4	8	IgG1	33%	100%
ID247	IgG2a	66	30	45%	0	6	IgG2a	100%	100%
ID269	IgG2a	205	3	1%	0	1	IgG2a	100%	100%
ID460	IgG1/IgG2b	126	59	47%	7	1	IgG2b	13%	100%

Table 1 | Results giving the pourcentage of IgG producers detected and harvested with the CellCelector system and the isotype specificity for anti-LRG1 clone candidates obtained(**a**)Number of fluorescent colony divided by the number of colony in MC(**b**)Number of colony producing anti-LRG1 divided by the number of colony picked(**c**)Number of colony producing anti-LRG1 divided by the number of isotype specific LRG1 colony picked

Summary

This application describes successful cloning of hybridomas in MC medium; the recovery of hybridomas after fusion under optimal conditions and rapid separation of the antibody specificities by distinguishing their isotype. After numerous clonings at BSI in MC medium using the CellCelector detection and picking technology, we routinely achieve 98% of clonality and 90% (+/- 5%) viability of the picked clones in liquid medium.

References

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