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REGULAR ARTICLE

High throughput production of mouse monoclonal antibodies using antigen microarrays

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Recent advances in proteomics research underscore the increasing need for high-affinity monoclonal antibodies, which are still generated with lengthy, low-throughput antibody production techniques. Here we present a semi-automated, high-throughput method of hybridoma generation and identification. Monoclonal antibodies were raised to different targets in single batch runs of 6–10 wk using multiplexed immunisations, automated fusion and cell-culture, and a novel antigen-coated microarray-screening assay. In a large-scale experiment, where eight mice were immunized with ten antigens each, we generated monoclonal antibodies against 68 of the targets (85%), within 6 wk of the primary immunization.

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1 Introduction

Although analysis of gene expression patterns using nucleic acid microarrays has become a powerful tool in genomic- and proteomic-scale studies, the results of these screens do not detect the presence of the expressed gene product, namely the protein. Only monoclonal antibodies and related affinity reagents detect the functional unit itself, and are therefore powerful and desirable tools in this detection process. Mouse-derived monoclonal antibodies continue to be the affinity reagent of choice in proteomics analyses, but their production against novel targets remains restricted by high tissue culture load and low-throughput screening methods [1]. Other techniques more amenable to high-throughput production of high affinity detection reagents are still unable to yield high affinity antibodies without lengthy downstream manipulation [2–8]. We identified two obstacles to increasing the mAb production throughput level. The first is the number of tissue culture operations necessary for performing multiple fusions simultaneously using only one antigen per animal. The second is screening the many thousands of culture supernatants generated by large-scale production.

Here we present a semi-automated method of hybridoma generation using mice immunized with multiple antigens and a novel antigen microarray assay (AMA), which simultaneously detects antigen-specific binding and determines the isotype of the bound antibodies. When tested using 80 antigens, our system isolated monoclonal hybridomas against 68 of the targets (85%) in a single batch run within 6 wk of primary immunization.

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Abbreviation: AMA, Antigen microarray assay

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2 Materials and methods

2.1 Immunization

BALB/c mice were immunized at 21-day intervals using 25 μg of antigen and boosted with 10 μg of antigen in Imject Alum (Pierce, IL, USA) with the addition of 10 nmols CpG-DNA [9] per mouse (TCC ATG ACG TTC CTG ATG CT, TIB MOLBIOL, Genova, Italy). Mice were bled 10 days after each immunization and serum titre tested by ELISA. The mice were additionally boosted 4 days prior to fusion using the same adjuvant and route of immunization.

2.2 Fusion

The spleen was rendered into a single-cell suspension by mechanical disruption. The suspension was filtered into a 50-mL tube (BD Falcon) through 70-μm nylon cell strainers (BD Falcon). The tube was centrifuged at $100 \times g$ for 10 min at room temperature (RT) and splenocytes resuspended in 5 mL Red Cell Lysis Buffer (Sigma, St. Louis, MO, USA) for 9 min at RT.

Hybridoma Medium HM20 (DMEM, 20% foetal bovine serum (Hyclone Defined), 10 mM L-glutamine, 50 μM Gentamicin) was added to a final volume of 50 mL and centrifuged for 10 min at RT with no brake. Supernatant solutions were aspirated to waste and cells resuspended in DMEM preheated to 37°C. Cells were washed twice more by steps of centrifugation and resuspension, and finally counted in a haemocytometer.

Separately, SP2 myeloma fusion partners (ATCC) were cultured for 5 days prior to fusion in HM20 and on the day of the fusion transferred to HM20/HCF/2xOPI (HM20 containing 10% Hybridoma Cloning Factor (Origen) and 2% OPI cloning supplement (Sigma)) for at least 1 h at 37°C in a 10% CO_2 humidified incubator. SP2 cells were washed three times in a similar fashion to the splenocytes and similarly counted. SP2 myelomas and spleen cells were mixed at a ratio of 1:5 (SP2:Spleen) and again centrifuged at $100 \times g$ for 10 min with no brake.

The supernatant was entirely aspirated to waste and Polyethyleneglycol 1500 in 50% HEPES (PEG: Roche Molecular Biochemicals) pre-heated to 37°C was pipetted drop-wise over 1 min with agitation to ensure even mixing. The cell/PEG mixture was incubated for 1 min at 37°C with gentle agitation. One millilitre of DMEM was added drop-wise over 1 min at 37°C with agitation. The mixture was further incubated for 1 min at 37°C with gentle agitation. A further 1 mL of DMEM was similarly added over 1 min at 37°C with gentle agitation and incubated for a further minute. Seven millilitres of HM20 was added drop-wise over 3 min at 37°C with gentle agitation. The tube was then spun at $90 \times g$ for 5 min with brake. The supernatant was aspirated to waste and the pellet resuspended in HM20/HCF/OPI/AH (HM20/HCF/OPI plus 10% Azaserine Hypoxanthine (Sigma)).

The post-fusion mixture was plated out into 20 96-well sterile plates (Nunc) at 100 μL/well and transferred to a humidified incubator (37°C, 10% CO_2).

On the third day after the fusion, the cells were fed with 100 μL HM20/HCF/OPI/AH. On day 7, culture supernatants were completely aspirated to waste and replaced with 150 μL of fresh HM20/HCF.

On day 11, 40 μL of each supernatant was transferred to 384-well plates (Greiner) as source plates for the microarray spotter.

2.3 Enzyme-linked immunoadsorbent assay (ELISA)

The 96-well plates (Maxisorp, Nunc) were coated with 4 μg/mL of antigen and incubated overnight at 4°C. The plates were washed in PBS 0.02% Tween-20 (PBST) and blocked with 3% BSA in PBS for 1 h at RT. Fifty microlitres of hybridoma supernatant was added to each well and incubated for 1 h at RT. After four washes in PBST, the plates were incubated for 1 h at RT with alkaline phosphatase conjugated anti-mouse secondary antibody, diluted 1:5000 in PBS (Jackson Dianova). Plates were washed in PBST and incubated with p-nitrophenyl phosphate (Sigma) for 10–15 min at RT. Reaction was stopped by adding 50 μL of 2 M NaOH and the OD was spectrophotometricly determined at 405 nm.

2.4 Microarray preparation

Aminosilane modified microscope slides (Aminosilane slides are generally available from EMBL-Heidelberg Genomics Core Facility (Genecore)) were homogeneously coated with 5 μg of antigen in 50 μL PBS using a 24×60 mm coverslip. Slides were incubated in a humid chamber at RT for 60 min, the coverslip removed and subjected to three 5-min washes in PBS. Slides were blocked in a 3% BSA solution in PBS for 60 min at RT. After five, 5-min washes in PBS, the slides were dried by centrifugation.

Hybridoma supernatants were spotted onto the slides using a MicroGrid II 600 arrayer, using 32 MicroSpot 2500 pins in an 8×4 array (Apogent Discoveries). Humidity and temperature were maintained at 40% and 24°C, respectively. Slides were left to incubate in the arrayer for a further 60 min. The microarrays were washed five times for 5 min in PBS and incubated with 40 μL of a mix of Cy3-conjugated goat anti-mouse IgG and Cy5-conjugated goat anti-mouse IgM (Jackson Immunoresearch, West Grove, PA, USA) both diluted 1:1000 in 3% BSA-10% Glycerol (60 min, RT, humid chamber). Microarrays were then washed twice for 5 min in PBST (0.2%), twice for 5 min in PBS and finally rinsed in ddH2O. Microarrays were dried by centrifugation and scanned in an LS400 Scanner (Tecan), using 633 and 543 nm lasers, respectively, for Cy5 and Cy3 excitation and 670 and 590 nm emission filters.

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2.5 Data analysis

Image analysis was performed using the GenePix Pro 4.1 software package (Axon Instruments). Spots for which the diameter is not included in a fork of 80–150 μm or of bad quality (scratches, heavy background, dust, *etc.*) were ignored. For each remaining sample, we retrieved the median of the medians of the intensities of each group of replicates (MR). Each value was then normalised against the median value of all the MR of the chip [MR/(median of total MR)]. Samples showing a normalised value of less than two were considered negative. Values between 2 and 20 were considered putative positives, while all samples having a normalised value equal or over 20 were considered positives. Data analysis was performed using a proprietary software application, Hy-CAT (Hybridoma Chip Analysis Tool).

3 Results

3.1 Multiplexed immunisation and microarray screening of hybridomas

To minimize the overall tissue culture load we first investigated the potential of immunizing mice with more than one target antigen and generating and isolating hybridomas that secreted antibodies, which would specifically recognize each one of the target antigens.

We immunized a single Balb/c mouse with five antigens (Table 1a). The mouse was boosted at 3-wk intervals and serum titre levels against each of the antigens were monitored by ELISA 10 days after each boost. When serum titre levels had reached a level where all were reactive by ELISA at a dilution of 1:2500, we harvested the spleens and fused with SP2 myelomas to form hybridomas using the standard protocols [10]. The

Table 1. a) Antigens details. Details of the antigens used for this immunisation experiments are here described. b) Multiple antigen immunization and AMA analysis for the production and isolation of monoclonal antibodies. Five antigens were used for the immunisation of a single mouse and the generated hybridoma library was screened against each antigen with AMA. Monoclonal antibodies were detected and obtained for all antigens tested. The number of positive hybridomas and their isotypes breakdown (columns 2, 3 and 4) are shown. ELISA screens confirmed the positivity of all the IgG isotype monoclonals selected (column 5). As the ELISA screen was performed on AMA IgG positives only, no ELISA IgM data is available

1a:

Antigen	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Expression method	Peptide/ protein	Species
Ago	PAZ domain of Argonaut 2	134.8 kDa	12.1 kDa	*E. coli*	Protein domain	*D. melanogaster*
Mago	Mago Nashi	17.3 kDa	17.3 kDa	*E. coli*	Protein	*D. melanogaster*
KetB4	Expressed fragment of Sls (2 Mda)	296 aa	34 kDa	*E. coli*	Protein	*D. melanogaster*
Tncf2	LiTnC1 (Troponin C isoform)	18 kDa	18 kDa	*E. coli*	Protein	*L. indicus*
PigMut4	Expressed fragment of Sls (2 Mda)	425 aa	48 kDa	*E. coli*	Protein	*D. melanogaster*

1b:

Antigen name	Total no. positive clones (AMA)	IgM secretors (AMA)	IgG secretors (AMA)	IgG secretors (ELISA)
Ago2	12	7	5	5
Mago JLY	17	8	9	9
KetB4	17	7	10	10
Tcnf2	29	9	20	20
Pig2Mut4	13	10	3	3

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fusion was plated into 20 96-well plates. Culture supernatants were harvested 12 days after the fusion for screening.

To maximize the screening throughput we developed a novel antigen microarray assay (AMA) and screened the fusion as follows: five aminosilane treated glass slides were coated each with 5 μg of one of the target antigens (one antigen on each slide). All of the culture supernatants were then spotted as a microarray onto each of the antigen-coated slides. The slides were subsequently incubated with a simple mixture of Cy5-conjugated anti-mouse IgM and Cy3-conjugated anti-mouse pan-IgG (recognizing all mouse IgG isotypes). After washing and scanning in a microarray scanner, we detected both IgG and IgM antibodies that bound specifically to each target antigen, the colour of the fluorochrome indicating the isotype of the bound antibody (Fig. 1). The microarray scanning results were cross-referenced by comparing each of the microarrays with each other,

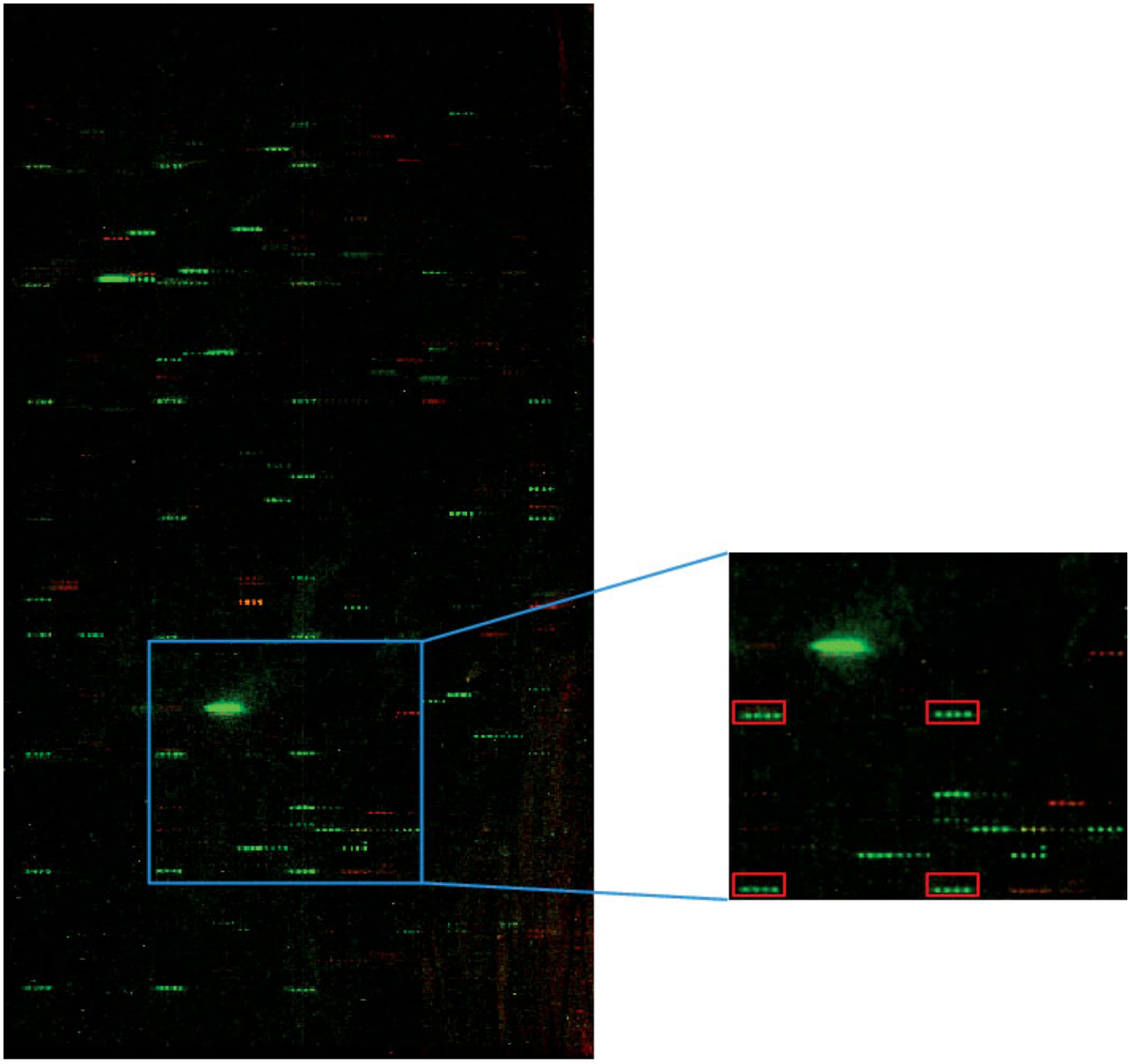

Figure 1. Scanned AMA chip. Scanned image of one AMA experiment. An aminosilane coated glass slide was coated with 5 μg of antigen and spotted with a library of 9,600 hybridoma supernatants. After a 60′ incubation in a humid chamber at room temperature, the array was hybridised with 50 μL of a solution containing Cy3-anti-mouse pan IgG and Cy5-anti-mouse IgM antibodies (diluted 1:1000). After washing, the array was scanned with a conventional microarray scanner. Red and green spots represent IgM and IgG monoclonal antibodies, respectively, specific for the coated antigen. Red boxes show the spots used as positive controls and sub-grid positioning aids. These consist of diluted blood samples from the immunised mice.

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and cross-reactive clones recognizing more than one target antigen were eliminated. Clones positive by AMA were further tested by ELISA and confirmed to be positive as shown also in Table 1b.

3.2 AMA-ELISA correlation study

Since only clones shown positive by AMA were further tested by ELISA, we investigated whether both positive and negative AMA results correlated with the ELISA. Five mice were immunized with nine antigens (Table 2a) and hybridomas were produced and analyzed by AMA and ELISA as already described. KetB5, Ket94/95, HMG CoA and His-IPAPB analysis show correlation values ranging from 76.81% to 96.22%. Even though the number of positives for each of these antigens was variable, ELISA OD values for these antigens were all over 1.0. CSD SAP (3 ELISA positives/2 AMA positives), 4950 (3/2), and IPAPB Pep2 (2/1), showed very low ELISA OD values. However, the sample size in both assays for these antigens was too small to be statistically significant. Nineteen IPAPBmid positive hybridomas were positive by ELISA even though their ELISA ODs were still close to the 0.2 cut-off value but only 11 were positive by AMA (Table 2b).

The AMA results show a high correlation with ELISA but the dynamic range of the HybriChips is greater than that of the ELISA, making the detection of positive samples with ELISA intensities near background levels more difficult and subjective (Fig. 2).

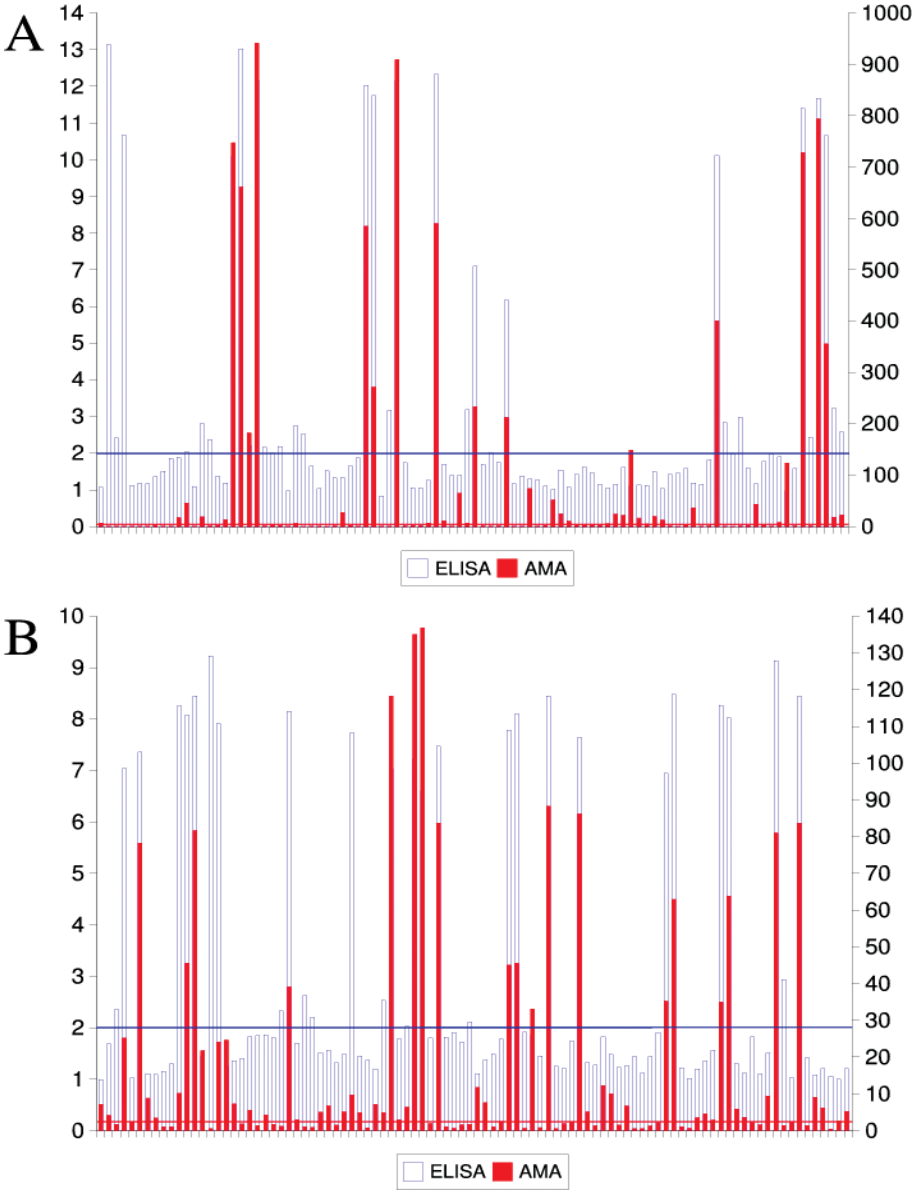

Figure 2. AMA-ELISA correlation. Correlation histograms for one representative 96-well plate for two of the antigens used for the trial, Ket94/95 (A) and KetB5 (B). Normalised values from the chip and the ELISA analysis were plotted in a sample-by-sample manner. HyrbiChip and ELISA normalised values are shown on the left and right axes respectively. The blue and red horizontal lines mark the "lower" normalised values required for a mAb to be considered positive on AMA and ELISA analysis, respectively.

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Table 2. a) Antigens details. Details of the antigens used for this immunisation experiments are here described. No information about the 4950 antigen was obtained from the investigators. b) Correlation study between AMA and ELISA screening methods. Nine antigens were used to immunize five mice. Each hybridoma line was screened by both ELISA and AMA. The table shows the results for both assays, where the "ELISA value range" field shows the lower and higher OD values of the positive antibodies and the "AMA/ELISA matches" shows the number of antibodies positive by both assays. The "total correlation" value was calculated as the ratio between AMA and ELISA positives. "Novel" samples are those antibodies that were positive by AMA, but negative in the ELISA screen

2a:

Antigen	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Peptide/ protein	Expression method	Species
GST Hupf	Hupf1	1,118 aa	1,398 aa	Protein	*E. coli*	*H. sapiens*
IPAPB-Mid	6xHis-inducible Poly(A) binding protein (middle fragment)	70 kDa	157 aa	Protein	*E. coli*	*H. sapiens*
His- IPABP	6xHis-inducible Poly(A) binding protein	70 kDa	71.6 kDa	Protein	*E. coli*	*H. sapiens*
IPABP-Pep2	inducible Poly(A) binding protein	70 kDa	15 aa	Peptide	Synthesised	*H. sapiens*
HMG CoA	HMG coenzyme A reductase	97.68 kDa	85 kDa	Fusion protein	*E. coli*	*H. sapiens*
CSD-SAP	Death inducer with SAP domain	1140 aa	63.5 kDa	Fusion protein	*E. coli*	*H. sapiens*
KetB5	Expressed fragment of Sls (2 Mda)	195 aa	22 kDa	Protein	*E. coli*	*D. melanogaster*

2b:

Antigen name	Mouse ID	ELISA positives	ELISA value range	AMA/ELISA matches	IgG	IgM	Total correlation	Novel
KetB5	540715	53	0.6052–1.6616	51	48	3	96.22%	4
Ket94	540715	15	0.7615–1.9997	13	13	0	86.67%	8
HMG CoA	1520	69	0.5094–1.9562	53	51	2	76.81%	0
CSD SAP	1520	3	0.2515–0.6053	2	1	1	66.00%	0
4950	540712	3	0.2508–0.4096	2	1	1	66.00%	1
GST-Hupf	540710	0	All <0.2	0	0	0	100.00%	0
His-IPAPB	540710	7	0.3042–1.2849	6	2	4	85.71%	0
IPAPB Pep2	540713	2	0.4048–0.4188	1	0	1	50.00%	0
IPAPB Mid	540713	19	0.1927–0.57	11	11	0	57.89%	0

3.3 Scale-up of the platform: 80 antigen trial

To test whether the AMA would be useful in a scaled up experiment we adapted and customized a commercially available robotics solution to perform eight fusions simultaneously and carry out downstream tissue culture. Eighty antigens were immunized into eight mice, ten in each animal (Table 3a, b). The immunization protocol was deliberately kept short to ensure broad-spectrum immunoreactivity and decrease immunofocussing and immunodominance [11]. After the primary immunization, the animals were boosted only once, on day 14, and the spleens were harvested on day 18. The fusion was robotically accomplished using standard protocols and the culture supernatants were subsequently harvested and screened by AMA. As shown in Table 3c, monoclonal antibodies were raised against 67 of the 80 antigens (83%) as tested by AMA. IgM secreting clones were raised against 32 (40%) of these antigens and we detected IgG secreting clones against 62 (77.5%) of the target antigens. Only IgG secretors were tested in further assays, and of these, hybridoma cell lines were isolated generating antibodies against 32 (40%) target antigens that were positive in at least one further immunoassay. Cell lines against

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Table 3. 80-antigen pilot test. a) Antigens details. Details of the antigens used for this immunisation experiments are here described. DWIR: "Data Withheld at Investigators Request" b) Individual results of the 80-antigen pilot test. Individual results are shown. For each of the 80 antigens used in the trial, the total number of specific hybridomas selected, their isotype and the number of these that showed ELISA positivity are listed. c) Summary and analysis of 80-antigen test results. All selected IgG species were screened by ELISA and Western blot. BlockA shows the number of antigens for which at least one mAb was detected by AMA and the number of ELISA and Western blot positives. Block B represents the breakdown of the results: the number of antigens for which antibodies were shown to be positive by AMA, ELISA and Western blot, by AMA and ELISA, by AMA and Western blot or by AMA only. Antigens for which no antibodies were selected or for which no Western blot was performed are shown in the two last columns of the table.

3a:

Antigen name	Owner	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Antigen origin
NK p46 D2	Dr. Mandelboim	NKp46	50 kDa	50 kDa	
NK p30	Dr. Mandelboim	NKp30	30 kDa	50 kDa	
NK AT8	Dr. Mandelboim	KIR2DS4	50 kDa	70 kDa	
DN 26	Dr. Averof	Af-apterous	unknown	280 aa	*A. franciscana*
Reverse gyrase	Dr. Stock	Reverse Gyrase	120 kDa	120 kDa	*A. fulgidus*
hGH	DWIR				
hGA	DWIR				
GST-Snu 13	DWIR				
GST-TgS1	DWIR				
GST-GRIP1	DWIR				
Vac ATPase	Dr. Stock	Vacuolar H+-ATPase	650 kDa (9 subunits: 64, 53, 35, 25, 20, 11, 13, 73 (TM), 8 (TM) kDa)	650 kDa	*T. thermophilus*
Smoothened	Dr. Therond	Smoothened	1031 aa	33 kDa	*D. melanogaster*
Hedgehog	Dr. Therond	Hedgehog	472 aa	25 kDa	*D. melanogaster*
CG33206A	Dr. Therond	D-GMAP210	1398 aa	302 aa	*D. melanogaster*
CG33206B	Dr. Therond	D-GMAP210	1398 aa	307 aa	*D. melanogaster*
Tace DOH CyT	DWIR				
GST-Tip 60	DWIR				
Grip1	DWIR				
GST-Hog1	DWIR				
CJ0601C	Dr. Lykke-Møller Sørensen	CJ0601c	49.7 kDa	49.7 kDa	*C. jejuni*
GST-Cofilin1 muscle	DWIR				
Gelsolin	DWIR				
Profilin2	DWIR				
12,6	DWIR				
RAC 1 V-12	DWIR				
Rho AV 14	DWIR				
Cdc42 Hs wt	DWIR				
Rab 27	Dr. Christoforidis	Rab27a	221 aa	221 aa	*H. sapiens*
Past 1/EDH	DWIR				
GST ADF	DWIR				
CHE alpha	DWIR				
Stat 1TC	DWIR				
CLIP 170 H2	DWIR				
PIST	Dr. Barr	PIST/GOPc	463 aa	475 aa	*H. sapiens*
SEC 18P	Dr. Ungermann	Sec18	66 kDa	66 kDa	*S. cerevisiae*
Yed Z-His	Dr. De Gier	YedZ	219 aa	211 aa	*E. coli*
CES1	DWIR				
DMAp	Dr. Averof	apterous	496 aa	60 aa	*D. melanogaster*
PA	DWIR				
SS	DWIR				

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Table 3. 3a: Continued

Antigen name	Owner	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Antigen origin
PopD	Dr. Dessen	PopD	295 aa	295 aa	*P. aeruginosa*
PcrV	Dr. Dessen	PcrV	294 aa	294 aa	*P. aeruginosa*
YID alpha sup12	DWIR				
ES1	Dr. Tocchini-Valentini	TRNA splicing endonuclease	182 aa	20 kDA	*S. solfataricus*
GST	DWIR				
POP	DWIR				
Profilin 1	DWIR				
FLN	Dr. Mosialos	Folliculin isoform 2	342 aa	62.7 kDa	*H. sapiens*
IK Beta	DWIR				
P3 fragment	Dr. Fankhauser	PIF4 (bHLH009)	430 aa	36.1 kDa	*A. thaliana*
GST cofilin2 non-muscle	DWIR				
SCAMP	DWIR				
EB-1	Dr. John	EB1	30 kDa	30 kDa	*H. sapiens*
UNC 59–61	Dr. John	Unc59, Unc61 complex	53 kDa each, tetrameric	53 kDa each	*C. elegans*
2C1	DWIR				
HIWI2	DWIR				
HILI	DWIR				
DNX	DWIR				
FABP	DWIR				
FADD	Dr. Ruberti	FADD	23 kDa	23 kDa	*H. sapiens*
KOC-1	DWIR				
TKT-L1	DWIR				
FADD-DD	Dr. Ruberti	FADD Death Domain	13.5 kDa	13.5 kDa	*H. sapiens*
cKet B2	Dr. Bullard	CketB2 (expressed fragment of SIs)	2 MDa	313 aa	*D. melanogaster*
Ket35/1	Dr. Bullard	Ket35–1 (expressed fragment of Kettin)	500 kDa	379 aa	*D. melanogaster*
Ket35/2	Dr. Bullard	Ket35–2 (expressed fragment of Kettin)	500 kDa	816 aa	*D. melanogaster*
Tncf 1	Dr. Bullard	TroponinC-F1	18 kDa	18 kDa	*L. indicus*
GST Tip1	Dr. Brunner	Tip1p	461 aa	50 kDa	*S. pombe*
LUMA peptide	DWIR				
YKt6	Dr. Ungermann	Ykt6	25 kDa	140 aa	*S. cerevisiae*
SHARP-SPOC	DWIR				
RXRb-LBD	DWIR				
SPD 1	Dr. Glotzer	SPD1	443 aa	443 aa	*C. elegans*
ZEN 4	Dr. Glotzer	ZEN-4	775 aa	434 aa	*C. elegans*
14–3-3 epsilon	Prof. Cesareni	14.3.3 epsilon	255 aa	255 aa	*H. sapiens*
14–3-3 zeta	Prof. Cesareni	14.3.3 zeta	245 aa	245 aa	*H. sapiens*
14–3-3 beta	Prof. Cesareni	14.3.3 beta	245 aa	245 aa	*H. sapiens*
P63 C-term	Prof. Cesareni	p63	641 aa	42 kDa	*H. sapiens*
POB 1	Prof. Cesareni	POB	511 aa	98 aa	*H. sapiens*
GST-Hrs	Prof. Cesareni	HRS	777 aa	280 kDa	*H. sapiens*

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Table 3.

3b:

Mouse	Antigen name	Total AMA positives	IgM positives (AMA)	IgG positives (AMA)	IgG positives (ELISA)
9721	NK p46 D2	6	4	2	1
	NK p30	13	7	6	2
	NK AT8	1	0	1	1
	DN 26	2	0	2	0
	Reverse gyrase	4	0	4	4
	hGH	2	0	2	0
	hGA	0	0	0	0
	GST-Snu 13	2	0	2	1
	GST-TgS1	1	1	0	0
	GST-GRIP1	1	1	0	0
9715	Vac ATPase	2	0	2	0
	Smoothened	0	0	0	0
	Hedgehog	0	0	0	0
	CG33206A	6	5	1	0
	CG33206B	4	0	4	4
	Tace DOH CyT	3	0	3	0
	GST-Tip 60	7	1	6	6
	Grip1	4	0	4	4
	GST-Hog1	0	0	0	0
	CJ0601C	0	0	0	0
9717	GST-Cofilin1 muscle	2	0	2	0
	Gelsolin	5	3	2	0
	Profilin2	2	1	1	0
	12,6	8	0	8	8
	RAC 1 V-12	3	0	3	0
	Rho AV 14	2	2	0	0
	Cdc42 Hs wt	7	3	4	1
	Rab 27	3	0	3	0
	Past 1/EDH	8	4	4	0
	GST ADF	5	2	3	0
9714	CHE alpha	2	1	1	0
	Stat 1TC	3	2	1	0
	CLIP 170 H2	0	0	0	0
	PIST	1	0	1	1
	SEC 18P	3	0	3	2
	Yed Z-His	12	0	12	0
	CES1	8	4	4	1
	DMAp	4	3	1	0
	PA	2	1	1	0
	SS	4	1	3	2
9716	PopD	12	0	12	10
	PcrV	2	1	1	0
	YID alpha sup12	10	0	10	8
	ES1	1	0	1	0
	GST	1	0	1	0
	POP	1	0	1	0
	Profilin 1	2	0	2	0
	FLN	0	0	0	0
	IK Beta	3	0	3	3
	P3 fragment	0	0	0	0
9718	GST cofilin2 non-muscle	2	0	2	0
	SCAMP	0	0	0	0
	EB-1	0	0	0	0
	UNC 59–61	5	0	5	0
	2C1	4	0	4	0

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Table 3. 3b: Continued

Mouse	Antigen name	Total AMA positives	IgM positives (AMA)	IgG positives (AMA)	IgG positives (ELISA)
	HIWI2	5	3	2	0
	HILI	4	1	3	0
	DNX	5	0	5	0
	FABP	4	2	2	0
	FADD	16	5	11	5
9719	KOC-1	8	5	3	0
	TKT-L1	3	1	2	0
	FADD-DD	0	0	0	0
	cKet B2	9	1	8	8
	Ket35/1	4	1	3	0
	Ket35/2	1	0	1	0
	Tncf 1	10	5	5	0
	GST Tip1	4	1	3	1
	LUMA peptide	2	0	2	1
	YKt6	2	0	2	2
9720	SHARP-SPOC	4	1	3	0
	RXRb-LBD	1	0	1	0
	SPD 1	0	0	0	0
	ZEN 4	7	2	5	4
	14–3-3 epsilon	7	1	6	0
	14–3-3 zeta	4	0	4	0
	14–3-3 beta	2	0	2	0
	P63 C-term	0	0	0	0
	POB 1	1	1	0	0
	GST-Hrs	1	1	0	0

3c:

BLOCK A					BLOCK B					
AMA positives	AMA IgM	AMA IgG	ELISA positives	WB positives	AMA/ ELISA/WB positives	AMA/ ELISA positives	AMA/WB positives	AMA only positives	Nega-tives	Missing WB data
9	4	7	5	2	2	3	0	4	1	2
6	2	6	3	1	1	2	0	3	4	0
10	6	8	2	2	2	0	0	8	0	3
9	6	9	4	5	4	0	1	4	1	1
8	1	8	5	6	3	2	3	0	2	2
8	4	8	1	4	1	0	3	4	2	3
9	6	9	4	1	1	3	0	5	1	4
8	5	6	1	1	1	0	0	7	2	0
67	34	61	25	22	15	10	7	35	13	15

15 of the 80 targets produced antibodies that were positive by both western blot and ELISA; 10 were positive by AMA and ELISA only, and seven positive by AMA and western blot only.

4 Discussion

We have presented a novel method for the high throughput production of mouse-derived monoclonal antibodies using multiplexed immunizations and a highly sensitive and parallel screening protocol based on antigen-coated microarrays. We have shown that it is possible to obtain specific monoclonal antibodies against each of the five antigens used for the multiplexing immunisation trial, and these observations were validated by several tests performed using different antigens. The novel screening method, which represents a major advance of the antibody production platform, allows for a quick and highly sensitive analysis of vast hybridoma libraries.

The disparity in the correlation between AMA and ELISA is due to two factors. The first factor is that the sensitivity of the AMA is higher than that of the ELISA

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(Tonkinson, J.L, http://www.devicelink.com/ivdt/archive/03/03/001.html) and that the threshold for what is considered positive by AMA was set too low. Indeed, when the threshold was increased the correlation between AMA and ELISA approached 100% (data not shown). The higher sensitivity of the AMA means also that factors such as low antibody concentration in the supernatant or the presence of an antibody of low binding affinity would give a positive result by AMA that would be negative by ELISA. Secondly, the conformation of the proteins on the different substrates could have been sufficiently different that antibody-binding sites on the protein that are available on the AMA substrate are masked by adsorbing the protein onto the polycarbonate substrate in the ELISA. Indeed this is borne out by the fact that seven of the antigens used in the 80-antigen trial (PcrV, ES1, GST, UNC59–61, HILI, HIWI, Yed-Z-His) were negative by ELISA and subsequently positive by western blot. It is also necessary to mention that the immunization protocol was shorter for the 80-antigen trial and the supernatants were harvested earlier compared to the experiments shown in Tables 1 and 2. We speculate that several species of monoclonal antibodies showing either lower affinity or lower concentrations were produced and thus fell below the threshold of detection for the ELISA.

Furthermore, AMA represents a qualitative improvement over ELISA as it allows for the selection of clones secreting antibodies with different isotypes directly at the primary screen using a mixture of up to five isotype specific secondary antibodies, each with a different flurochrome. ELISA can also be used to determine the isotype of monoclonal antibodies but this is however more laborious requiring up to five separate ELISAs per hybridoma (IgM, IgG1, IgG2a, IgG2b, IgG3). IgM isotypes are often more useful to investigators for fluorescence-based assays such as FACS analysis or immunofluorescence, but this isotype is almost useless for assays involving protein-A and protein-G as detection reagents, most notably immuno-electronmicroscopy and immunoprecipitation, due to their lack of affinity for protein-A or protein-G. IgM isotype antibodies are also notoriously difficult to purify and in these two cases IgG isotypes are more desirable.

In the 80-antigen pilot study we achieved a success rate of 40% for antibodies that worked in at least one other immunoassay, or 28% for antibodies which were functional in at least one immuno-application. Comparison with similar studies is difficult, as others have focused on the production of antibody serum titre [12–14] as opposed to the isolation and production of hybridomas, however when compared to the best of these serum-titre studies [12], our method is about half as successful as the 80% success rate achieved there.

Improvements in these success rates may be possible by slightly lengthening the immunization protocol. The longer immunization protocols used in the experiments represented by Tables 1 and 2 may well account for the higher efficacy over the 80-antigen experiment. It is also possible that increasing the number of immunogens for each animal may ultimately lower the number of positive clones in any one fusion. Indeed, subsequent production runs within our laboratory have shown that five antigens per animal is optimal, however recent improvements in immunization strategies have allowed us to immunize using ten antigens with higher success rate than in the 80-antigen experiment (data not shown). Using genetic immunization protocols may prove even more effective in attaining a suitable response against a higher proportion of the target antigens [12].

In summary, we have developed a fast, economical, high-throughput method for hybridoma generation, which improves upon the conventional, non-automated system in the following ways. Firstly, the turnaround time from receipt of the antigen to delivery of cells to the investigator has been reduced from 4 to 6 months to around 2 months. Secondly, there is a cost reduction of approximately five-fold (in the case of five antigens per animal) per target. Thirdly, the throughput has increased from approximately 20 specific target antigens per capita per annum to 150 specific target antigens per capita per annum. Further increases in throughput would be possible by automating the downstream tissue culture (including clonal expansion and freeze down steps), a bottleneck which was not addressed in this study. A simple scaling up of the methodology proposed here along with the above-mentioned automation of the clonal expansion and freeze down, could help to alleviate the current restrictions on the availability of these important affinity reagents.

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