A 96-Well Plate Assay for Relative Monoclonal Antibody Titers

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Monoclonal antibodies are used to treat a wide range of illnesses such as rheumatoid arthritis, Crohn's Disease, and follicular non-Hodgkin lymphoma (**1**). The identification and use of certain growth medium additives and modified culture conditions during cell culture processes that produce MAbs may lower production costs—which could help lower the overall costs of those drugs. Additives such as dimethyl sulfoxide, sodium butyrate, and rapamycin have been reported to increase MAb titers by 30–120% in various cell culture systems (**2–5**). Changes in culture conditions such as temperature and osmolality are also shown to influence MAb titers to similar extents in certain cell culture systems. For example, increasing culture temperature from 33 °C to 39 °C more than doubled specific MAb production (from 0.07 to 0.15 × 10^{-9} mg/cell/h) in one case, whereas increasing osmolality of the medium (from 286 mOsm/kg to 398 mOsm/kg) caused a 55% increase in specific MAb productivity for another (**6, 7**).

Unfortunately, the effects of adding compounds or changing conditions on MAb productivity and titer are variable and unpredictable for any given cell culture system. One example is the reported 1.4-fold increase in production of a recombinant protein for a culture of Chinese hamster ovary (CHO) cells in the presence of 1% DMSO, conditions that suppressed MAb production in three hybridoma cell lines (**8**). In another work, just two of five hybridoma cell lines tested with retinyl acetate showed a significant increase in MAb titer (**9**). And switching from a serum-containing to a serum-free medium affects productivity unpredictably. In one case, the switch led to an increase in MAb titer from 3.5 µg/mL to 9.0 µg/mL (**10**).

Highly variable and unpredictable cell line responses complicate selection of enhancers and optimization of cell culture conditions. Thus, a methodology is needed for quickly and efficiently assessing how reported enhancers and/or modified culture conditions will influence the MAb titer in a particular cell line or newly created clone under investigation. Presumably there are more compounds and conditions that could serve to increase MAb titers, and quickly identifying them would be useful.

Our objective was to demonstrate a high-throughput capability for identifying statistically significant differences in 72-hour MAb titers for test and control conditions. To this end, we developed a 96-well plate assay for relative MAb titers that consists of a 72-hour batch culture followed by a MAb ELISA. Our assay is demonstrated for control hybridoma cultures and those provided with select concentrations of rapamycin (**5**), sodium butyrate (**3, 4**), dimethyl sulfoxide (**2, 8**), and sodium chloride.

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PRODUCT FOCUS: MONOCLONAL ANTIBODIES

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LEVEL: INTERMEDIATE

MATERIALS AND METHODS

Cell Culture: For all experiments we used nonadherent murine hybridoma cells from ATCC (www.atcc.org, catalog number CRL1606) that secrete an immunoglobulin (IgG) against human fibronectin. During routine maintenance, cells were subcultured daily at 2.5 × 10^5 viable cells/mL and

maintained in T-flasks from Corning Inc. (www.corning.com) that were placed in a Model 3100 series incubator from Forma Scientific (www.forma.com) and controlled to 37 °C, 95% humidity, and 10% CO_2. The serum- and hydrolystate-free medium we used for maintenance and 96-well plate MAb titer assays consisted of glutamine-free IMDM from Mediatech (www.cellgro.com) supplemented with 4.0 mM glutamine (also from Mediatech) and from Sigma (www.sial.com),

- 10 mg/L insulin
- 5 mg/L holo-transferrin
- 7.5 mg/L protease-free BSA
- 2.44 µL/L 2-aminoethanol
- 3.5 µL/L 2-mercaptoethanol
- 10 units/mL penicillin
- 10 µg/mL streptomycin.

Test Chemicals and Working Stock Solutions: All chemicals came from Sigma and were 95% pure or better. DMSO (catalog number D-2650) and NaCl (catalog number S-9625) were used as is and stored at 25 °C. A stock solution of 873 mM NaBu (catalog number B-5887) was prepared in sterile tissue culture grade water. A stock solution of 1 mM rapamycin (catalog number R-0395) was prepared in DMSO. We prepared a resazurin reduction assay reagent at a concentration of 0.0105% in sterile tissue-culture water and then filtered it.

96-Well Plate Assay for Relative MAb Titers: Each assay consisted of a 72-hour batch culture in a 96-well plate from Nunc (catalog number 149026), followed by a MAb ELISA. Four concentrations of each chemical (Table 1) were tested in parallel within columns 2–5 and 6–9 on the same plate. Column 1 was charged with 50 µL of test-compound–free medium, columns 2–9 were charged with medium containing a certain concentration of chemical, and columns 10–12 remained empty. All liquid handling steps were accomplished using e-line brand eight- or 12-channel electronic pipettors from Biohit Inc. (KSE Scientific, www.ksesci.com).

Next, we added 50 µL of cell culture (concentration = 4.0×10^5 viable cells/mL) to the wells of columns 1–9. This cell culture came from a parent T-flask that had been counted using Trypan Blue Exclusion, then centrifuged, and then resuspended in medium at the desired viable cell concentration. We incubated the batch culture plate at 37 °C, 95% humidity, and 10% CO_2 for 72 hours. One hour before the end of this culture incubation, 10 µL of resazurin reagent solution was added to each well culture.

After culture incubation, we read the fluorescence of each well at wavelengths of 530 and 590 on a FL600 plate reader from Bio-Tek Instruments (www.biotek.com) and then centrifuged the cultures at 200*g* for 10 minutes within the same plate. Next, 10-µL volumes of supernatant were transferred directly into a second 96-well plate, the MAb storage plate. It was prepared just before that with 190 µL of diluent solution: one part SuperBlock blocking solution (catalog number 37515 from Pierce Chemical Company, www.piercenet.com) and nine parts phosphate buffered saline (catalog number P-4417 from Sigma). The MAb storage plate was covered with paraffin film and placed in a refrigerator at 4 °C until needed for the ELISA (within one hour).

Starting at hour 70 of the culture incubation, we coated each well of a 96-well Nunc Immuno Plate ELISA plate from (C96 Maxisorp certified)

Table 1: Preparation of test media used in the MTA experiments; concentrations shown are those after the twofold dilution. Note that preparation of NaBu and Rap involved intermediate steps.

Chemical	Concentration	Amount of Stock*	Medium (mL)
DMSO (% by volume)	0.05	5.93	5.99
	0.10	11.85	5.99
	0.20	23.70	5.98
	0.30	83.19	5.96
NaCl (mM)	3.45	0.0041 g	10.00
	6.90	0.0081 g	10.00
	13.90	0.0162 g	10.00
	20.80	0.0243 g	10.00
$NaBu_{INTER}$ (mM)	21.30	250.00	10.00
NaBu (µM)	250.00	234.70	9.77
	312.00	293.40	9.71
	625.00	586.70	9.41
	700.00	657.70	9.34
Rap_{INTER} (µM)	9.94	1.00	9.90
Rap (nM)	45.00	91.00	9.91
	58.50	118.00	9.88
	131.70	265.00	9.74
	150.00	302.00	9.70

* in µL unless otherwise stated

Abbreviations Used Herein

ANOVA: analysis of variables

BSA: bovine serum albumin

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

HSD: Tukey–Kramer honestly significant difference test

IMDM: Iscove's modified Dulbecco's medium

MAb: monoclonal antibody

MSD: minimum significant difference

MS_{within}: mean square within a group

MTA: a 96-well plate assay for relative monoclonal antibody titers

NaBu: sodium butyrate

NaCl: sodium chloride

OD: optical density

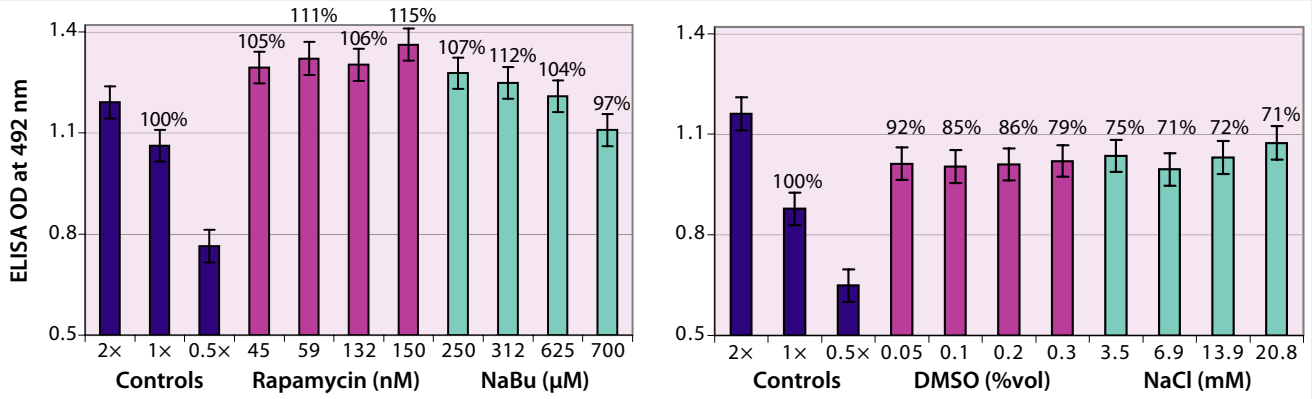
PBS: phosphate-buffered saline

Rap: rapamycin

SE: standard error

SMA: sublethal metabolic activity

Figure 1: Relative MAb titers and viable-cell concentrations for 72-hour batch hybridoma cultures with and without various concentrations of (LEFT) Rap or NaBu or (RIGHT) DMSO or NaCl. Bars represent means for ELISA ODs ± 0.5 × MSD, and numbers above them show the final relative viable-cell concentrations, whose MSDs were 17% (LEFT) and 8% (BOTTOM). Sample sizes for MAb data left-to-right: (LEFT) n = 13, 14, 13, 14, 12, 13, 14, 15, 14, 14, and 14; (RIGHT) n = 13, 14, 14, 14, 13, 15, 16, 15, 14, 13 and 12. Sample sizes for viable cell concentration data left-to-right: (LEFT) n = 14, 13, 14, 14, 14, 14, 15, 14, and 15; (RIGHT) n = 14, 14, 14, 14, 14, 13, 13, 14, and 13.



from VWR Scientific Products (www.vwrsp.com) with 50 µL of 8.0-µg/mL of human fibronectin (catalog number HFBN-804 from Innovative Research, Inc., www.innov-research.com) in Tris-HCl coating buffer (Pierce BupH Tris buffered saline pack, catalog number 28379). That plate was then incubated for two hours at room temperature, with 60 rpm orbital shaking using the model 57018-54 VWR orbital shaker under humid conditions.

Next, we removed the fibronectin and coating buffer and blocked the plate with pure blocking solution, then washed it with PBS. We added diluent solution to the wells as follows:

- 50 µL to column 1,
- 45 µL to columns 2–9 and 11,
- 40 µL to column 10
- 47.5 µL to column 12.

Then 5-µL volumes of 20×-diluted culture supernatant samples from the MAb storage plate were transferred into columns 2–9 of the ELISA plate for a total dilution of 200×. And 10-µL, 5-µL, and 2.5-µL volumes of supernatant were transferred from the wells of column 1 on the MAb storage plate (our control culture) into the wells of columns 10–12 of the ELISA plate. Those served as 100×, 200×, and 400× dilutions of the control.

The plate was then incubated at room temperature for an hour, with orbital shaking at 60 rpm. We removed the MAb/diluent solution from the ELISA plate and washed it using our PBS washing solution. Then 50 µL of 0.4-µg/mL goat anti-mouse IgG(Fc)-HRP detection antibody from US Biological Inc. (www.usbio.net, catalog number I1904-14C) was placed in each well of the ELISA plate and incubated with shaking for an hour.

After removing the detection antibody solution and washing the plate, we added 50 µL per well of Sigma Fast OPD (catalog number P-9187), wrapped the plate in foil, and incubated it for 15 minutes at room temperature with orbital shaking at 90 rpm. The reaction was halted by addition of 50 µL 1M HCl (catalog number 920-1 from Sigma) to each well, and the plate was then incubated for another four minutes with shaking. Optical densities of the wells were measured twice at the 492-nm wavelength using a µ-Quant spectrophotometer from Bio-Tek Instruments.

Data Analysis: We removed outliers within each trial (n = 8 for each condition of a single trial) if they fell outside the 98% confidence interval, using the standard error from the MS_{within} groups of a one-way ANOVA. Remaining values for each condition were normalized by the average value of their control and re-scaled by the average value of controls from both trials, yielding $n \leq 16$ for the ELISA or resazurin data of each condition tested. Differences in pairs of means were analyzed by two-way ANOVA and the HSD test for statistical significance at a confidence level of 95% (**11**). Visual approximation of the significance of differences between means is shown with error bars equal to one-half the average MSD.

Results

We compared MAb titer differences at 72 hours in batch culture by comparing mean ODs from the final step of our MAb ELISA for the three controls and eight conditions using a test compound (Figure 1, blank not shown). Each result is the mean of OD values for supernatant samples from the 12–16 replicate cultures for a given condition, with outliers excluded. The error bars are comparison limits, which approximately indicate statistical significance of differences for each pair of means when they do not overlap.

We first compared test conditions and the 1× control, all of which had supernatant samples diluted 200-fold in the ELISA. In Figure 1, it is evident that all test conditions with Rap, NaBu, DMSO, or NaCl (except for 700 µM NaBu) yielded larger ODs than the 1× control. This signifies that those cultures produced more MAbs than the control culture did, results which are significant at the 95% confidence level.

A second set of comparisons was made between four conditions given the same chemical. Here it is evident that none of the differences in MAb titers was statistically significant for the Rap, DMSO, or NaCl groups. Differences in MAb titer among conditions with concentrations of 250, 312, and 625 µM NaBu were not significant, whereas all three were

significantly different from 700 µM NaBu.

Having tested two chemicals in parallel in each MTA experiment, we performed a third set of comparisons between cultures given Rap or NaBu and those given DMSO or NaCl. For Rap and NaBu, seven of 16 comparisons had differences that were statistically significant. All conditions with Rap had MAb titers greater than that of the 700-µM NaBu condition, whereas none were different from the 250-µM NaBu. For DMSO and NaCl, none of the eight conditions yielded significantly different MAb titers.

In a fourth set, we compared the MAb ELISA ODs for each chemical concentration with two additional controls: one from control culture supernatant prepared at 400× dilution (hence 0.5× of the 200× control) and the other at 100× dilution (hence 2× of the 200× control). These provided a rough means to assess the magnitudes of observed differences. Mean ODs for cultures with Rap were all greater than the 2× control, and these differences were all statistically significant. Mean ODs for cultures with NaBu spanned a range of slightly greater than 1× to slightly greater than 2×, yet none of differences from the 2× control were statistically significant. Mean ODs for cultures with DMSO and NaCl fell between those for 1× and 2× controls. Differences of all from 1× and from 2× were statistically significant, except for that of 20.8 mM NaCl with 2×.

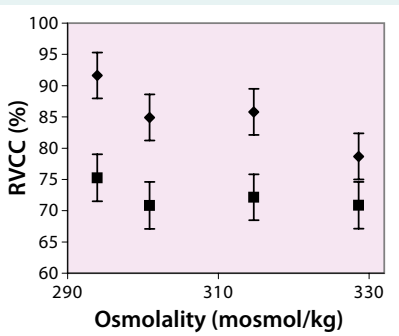
We also obtained final viable cell concentrations relative to control cultures during MTA screens (shown as percentages in Figure 1). None of the differences were statistically significant here between control culture and those given Rap or NaBu, although many values were 4–15% greater. Differences within either chemical group were insignificant. Just one of 16 comparisons across groups (Rap and NaBu) was statistically significant: that for 150-nM Rap (115%) and 700-µM NaBu (97%).

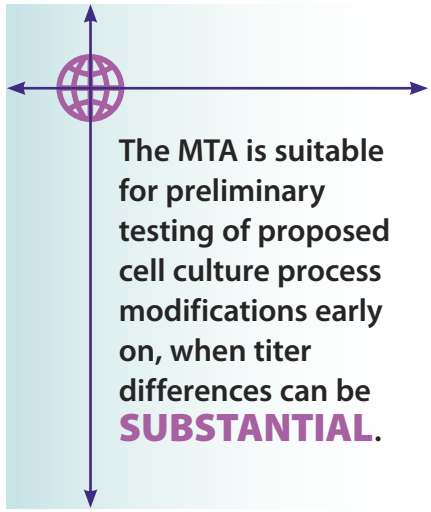
All differences in final relative viable cell concentrations between control cultures and those given DMSO or NaCl were statistically significant, with test values being 8–29% lower than control. Differences within chemical groups were insignificant, with the exception of that between 0.05 %vol (92%) and 0.3 %vol DMSO (79%). Of 16 comparisons across groups (DMSO vs. NaCl), 14 were statistically significant, with relative viable cell concentrations for 0.3 %vol DMSO (79%) not statistically different than either 3.5 or 13.9 mM NaCl (75% and 72%, respectively).

We distinguished the effect of DMSO on MAb titer enhancement from an osmolality effect by comparing MTA results for cultures given NaCl at concentrations yielding the same increases in osmolality. DMSO concentrations of 0.05% vol, 0.1% vol, 0.2% vol, and 0.3% vol correspond to 6.9 mM, 13.9 mM, 27.7 mM, and 41.6 mM, respectively. We estimated theoretically the contributions to osmolality of culture medium by addition of DMSO (assuming no disassociation of DMSO, hence 1 mOsm for each mM DMSO) and confirmed them experimentally with a Model 3D3 osmometer from Advanced Instruments, Inc. (www.aii2.com), but those data are not shown here. The relative MAb titer results for DMSO and NaCl were no different, but the relative final viable cell concentrations were (Figure 1–RIGHT).

Grouping the data in pairs by similar initial osmolality highlights the difference in outcomes with DMSO and NaCl (Figure 2). All relative viable cell concentrations were greater with DMSO than with NaCl, and all differences were statistically significant. So the effects of DMSO and NaCl on growth and death of cells was different. Additionally, given similar MAb titer outcomes, the specific productivities may have been different, depending on unobserved viable cell concentration profiles during the batch. DMSO may possibly benefit from an osmolality effect, but its effect on viable cell concentration (and possibly specific productivity) appeared to differ from that of osmolality increase resulting from having added NaCl.

Figure 2: Final relative viable-cell concentrations (RVCC) compared with initial osmolality for 72-hour batch hybridoma cultures using DMSO (diamonds) or NaCl (squares). The error bars are ± 0.5 × MSD. Sample sizes for DMSO: n = 14. Sample sizes for NaCl: n = 13, 13, 14, and 13 (left to right).

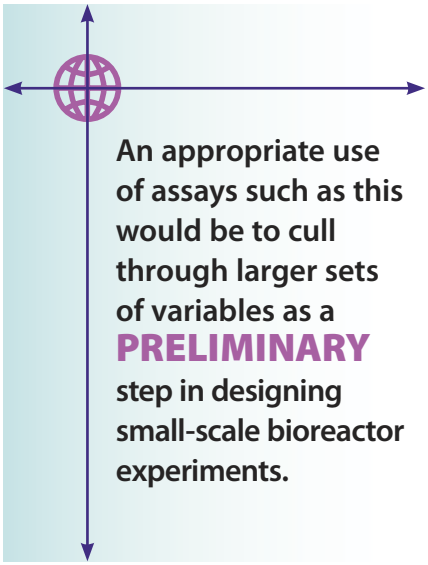




Discussion

Our demonstrations of the 96-well plate assay for relative MAb titers were made using molecules and conditions already reported to improve MAb titers. That is the primary reason 15 of 16 test conditions yielded titers that were greater than the control's. Also, we conducted 18-hour 96-well plate assays for sublethal metabolic activity (results not shown) beforehand to verify that chosen concentrations were nontoxic and that at least one was within the metabolic effect range (**12**). For Rap and DMSO (and for NaCl as an osmolality control), we essentially tested concentrations quoted in literature as effective. For NaBu, the SMA results for toxicity were used to exclude concentrations above 700 µM.

The MTA's level of precision and use of within-experiment relative controls make it suitable for preliminary testing



of proposed modifications to cell culture processes during early phase process development, when titer differences can be substantial. A statistical comparison of ODs against three controls can coarsely separate changes in MAb titer into seven statistically distinct groups:

- titer < 0.5×
- titer = 0.5×
- 0.5× < titer < 1×
- titer = 1×
- 1× < titer < 2×
- titer = 2×
- titer > 2×.

Alternatively, the statistical analyses provided minimum significant differences in ELISA ODs of 0.095 (for Rap and NaBu) and 0.097 (for DMSO and NaCl). These differences translate to 36% and 30% changes in MAb titer, respectively, based on the slopes observed for the three controls (m = 0.7078 and 0.8505, respectively, for equation OD = m × logC + b). That level of precision was sufficient to discern differences both between test titers and controls and within a test group (e.g., 700-mM NaBu compared with lower concentrations). Running two trials helped block variance due to inoculum variation and served to increase precision slightly (e.g., results for individual trials with Rap and NaBu provided statistical discrimination of MAb titers of ±40–45% as opposed to ±36%).

The low cost and high-throughput format also support the MTA's use in preliminary testing of proposed modifications. In our laboratory, performing a single MTA cost about $55 in supplies and required about 80 hours' time, about eight of which were dedicated hours. That was repeated four times to yield the data presented herein. Both costs and time required were lower than those needed for doing similar work in T-flasks or bioreactors.

The increase in throughput, lowered cost, and decreased labor are not without a trade-off, however: 100-µL sized cultures may not exactly represent process conditions. So an appropriate use of assays such as the MTA would be to cull through larger sets of variables as a preliminary step in designing small-scale bioreactor experiments. That could save time and money on subsequent process development steps, not to mention in identifying new supplements or conditions that ultimately could help make more MAb product.

Additionally, for novel modifications without precedent, a 96-well plate assay for sublethal activity may be used before the MTA because it costs about $55 and takes only 18 hours, about four of which are dedicated time. We found such foreknowledge from an SMA screen to be helpful in our work when it came to choosing NaBu concentrations from reports that quoted a four-decade range of efficacy in various other cell lines.

Our 96-well plate assay for relative MAb titer is a low-cost, high-throughput test for changes in relative MAb titers of ±33% or greater during 72-hour mammalian cell batch cultures. Compounds we tested were well-known for enhancing MAb titer, and our assay confirmed that in a hybridoma cell line. Cost-effective assays such as those described here should be useful in testing large numbers of variables during preliminary, early phase process development. Variables could include changes in medium formulation, cell line or clone selection, and additive type and concentration.

Acknowledgments

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