



CELLine Technical Report IV

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CL 6-well-Experimental screening device
Application: Murine and Rat Hybridoma

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Cell culture in hollow fiber systems and dialysis based mini fermentors has become more prevalent due to the advantages provided by separating cells from nutrient medium with a semi permeable membrane (1-5). These advantages include a marked reduction in serum use, concentration of cell secreted products and higher cell densities. A limitation to these systems is the difficulty usually associated with experimental evaluation of different culture conditions. Most of these systems are relatively large scale and consume significant amounts of medium and time per individual culture. The ability to set up multiple cultures and examine various conditions simultaneously is limited. Due to these difficulties, analysis of parameters which can affect performance of cultures is often based on limited replicates and focused on few experimental conditions. In this report, a simple multiple chamber culture plate (CL 6-well, INTEGRA Biosciences, Ijamsville, US) was tested to determine its suitability as a screening device.

Addition of serum in the perfusate or nutrient medium separated from cells by a semi permeable membrane has been reported as being beneficial (6,7). Removal of serum entirely from the perfusate or nutrient medium however has also been shown to be successful (8). The impact of serum components which may cross the semipermeable membrane and impact cell growth or production is not well defined.

An additional feature of many dialysis based cell culture systems is the need for a minimum cell inoculum. A lag phase in cell growth following inoculation is seen in many systems and can reduce production rates until sufficient cell growth begins (7,8). Determination of factors which may influence this initial growth phase would allow for a strategy that would reduce the time required to achieve maximum production rate (9). Screening of various culture conditions to assess impact on initial cell outgrowth would be beneficial if a more rapid start up of the culture could be achieved.

To determine the effect of medium supplementation on cell growth and antibody production in a dialysis based cell culture system, triplicate cultures were set up under different experimental conditions in CL 6-well (INTEGRA Biosciences, Ijamsville MD) culture plates. The CL 6-well has 6 independent cell compartment/nutrient medium reservoirs and allows 6 small scale cultures to be conducted simultaneously within a single device. The cells are cultured in a cell compartment, separated by a 10,000 MWCO semipermeable membrane, from the nutrient medium reservoir. As many of the principles of hollow fiber culture systems are reproduced in the CELLine culture devices, they should be well suited for experimental testing of nutrient medium supplementation and its effects on high density cell growth and production. Importantly, they should allow for comparison of antibody production between low density and high density cultures.

Cells: To assess response variation which may be cell line specific, 7 different hybridoma cell lines were evaluated. 3 murine hybridoma cell lines and 4 rat hybridoma cell lines (Table 1) were used. The 7 cell lines produced different amounts of antibody per ml of culture supernatant in spinner flask culture. The highest producer (MH7.0) yielded 15 ug/ml of antibody in spinner culture supernatant. Two rat hybridomas (RH525, RH 526) did not produce antibody at a detectable level (<1 ug/ml) in spinner culture. All cell lines except for RH525 and MH7.0 are available from American Tissue Type Collection (ATTC).

The cells were thawed from frozen stocks, and following demonstration of cell proliferation in static culture, were propagated in small spinner flasks (100ml) to determine growth rates, production rates, and maximum cell densities. Cells were cultured in IMDM with supplements and 10% FBS for growth curve analysis. Antibody concentration was measured in culture supernatant at end of growth phase. For inoculation into the CL 6-well, cells were harvested from cultures in growth phase and with cell viability greater than 85%. Cells were centrifuged and resus-



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pended in fresh cell compartment medium at a concentration of 1.5×10^6 cells/ml and inoculated (800 μ l) into the cell compartments in triplicate wells of multiple CL 6-well plates. Nutrient medium was placed in the nutrient reservoir of each cell compartment (25 ml) except for Group 5

in which 5 ml of nutrient medium was added followed by additional 10 ml on day 3 and day 5 bringing total volume to 25 ml. Culture plates were placed into a 37°C humidified 5% CO₂ incubators.

Table 1

	MH 70	MH437	MH479	RH525	RH526	RH527	RH528
ATCC No.	none	AB-27	HB-9713	none	HB-8794	HB-188	CRL-2226
Isotype	IgG1	IgG2a	IgG2b	IgG	IgG2a	IgG1	IgG2b
Fusion Partner	NS-1	Sp2/O-Ag14	P3X63Ag.8 U!	n.a.	Sp2/O	P3-Ns1/Ag4-1	Sp2/O-Ag14
Static Production (μ g/ml)	15	11	12	<1	<1	4	14
Max. viable cell concentration x 10^6 /ml (spinner culture)	1.6	1.1	1.1	1.3	0.9	0.7	0.5

Cell compartment medium: IMDM: 2X L-glutamine (5 mM), penicillin G (66 mg/L) streptomycin sulfate (144 mg/l), contained either 10%, 15% or 20% FBS as a growth supplement.

Nutrient medium: IMDM:2X L-glutamine (5 mM), penicillin G (66 mg/L) streptomycin sulfate (144 mg/l) was supplemented with either 0%, 1% FBS. Additional supplementation of nutrient medium with ethanolamine(Sigma, St.Louis, MO) or a mixture of non-essential amino acids was also evaluated.

Experimental Conditions are shown in Table 2. Different concentrations of FBS supplementation to either the cell compartment medium or the nutrient medium was evaluated. A stepped nutrient feed strategy was evaluated in Group 5 (nutrient medium provided at Day 0 was reduced and increased step wise to determine if dilution of cell compartment contents by excess nutrient medium at inoculation influenced initial outgrowth from inoculum). Group 6 was supplemented with ethanolamine (4 mg/ml, SIGMA, St.Louis MO) which has been reported to be stimulatory to hybridoma cell growth and production (10). All of the cell lines were cultured under the experimental conditions in triplicate.

Table 2

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Cell Compartment	15% FBS	15% FBS	20% FBS	20% FBS	15% FBS	10% ethanolamine
Nutrient Medium	0% FBS	1% FBS	0% FBS	1% FBS	0% FBS stepped feed	0% ethanolamine



Harvest: Cell compartment contents were split back on days of culture indicated. The volume in the cell compartment was drawn up into a 1 ml graduated pipette and the volume recorded. 400 μ l of the measured cell compartment volume was returned to cell compartment, and the remaining volume (400-500 μ l) was harvested for ELISA and cell counts. A residual volume of approximately 100 μ l was retained in the cell compartments and could not be withdrawn due to geometry of cell compartment. Residual volume was determined by placing 800 μ l of volume into cell compartments followed by measurement of volume upon immediate withdrawal. The mean volume retained within a cell compartment was determined to be approx. 100 μ l (multiple measurements). The residual volume was trapped between the two membranes of the cell compartment and in the inlet to the cell compartment. The residual volume (100 μ l) was added to the volume measured by pipette withdrawal, from the cell compartments when cell compartment volume was determined. The harvested cell containing supernatant fraction was kept sterile and stored at 4 °C until ELISA determination. Fresh cell compartment medium (400 μ l) was added to the cell compartment to replenish the harvested cell compartment volume. Cell numbers in the samples removed from the cell compartment were determined by diluting and counting with a standard hemacytometer. Viable cells were discriminated from non-viable cells by trypan blue staining and phase contrast microscopy. Nutrient medium was removed from each reservoir and replaced with fresh nutrient medium (25 ml) when cell compartment was harvested. Experimental conditions were maintained for the duration of the cultures.

ELISA: Sandwich ELISA was performed with polyclonal goat anti-mouse IgG or IgM capture antibody and polyclonal anti-mouse IgG or IgM antibody labeled with peroxidase. Color was developed with ABTS. Dilution curves for each sample were ran and the values which fell within the linear range of the standard curve (the last three values) were averaged to obtain ELISA data.

Results: The concentration of total cells (mean , n=3) per ml of cell compartment volume for each experimental group on harvest days are shown for each cell line in [Fig.1](#). The splitting back of the cell compartment on the harvest day is shown by the vertical lines connecting the total numbers at harvest and the reinoculated numbers. Cell growth following harvest was demonstrated by the

increase in cell numbers at the next harvest. In general the numbers of total cells increased with culture duration. There were no consistent differences seen between experimental groups with regards to the total cell concentration measured in the cell compartments. Cell growth as indicated by total cell concentrations measured for triplicate cultures in an experimental group for a particular cell line, did not reveal any consistent benefit associated with the various supplementation approaches.

As shown in [Fig. 2](#), the viable cell concentrations measured in triplicate for the different experimental groups for each cell line also did not demonstrate any consistent effect associated with the various supplementation strategies. The viable cell concentrations in the cultures tended also to increase with the duration of culture. Maximum viable cell numbers were not attained 7 days post inoculation except for the MH7.0 cell line. The other cell lines required additional time of culture before reaching maximum viable cell numbers. The cell line RH528 had increasing viable cell numbers up to day 22 of culture. Differences between experimental groups were seen on a particular harvest day (RH527 Day 7), in some instances, however the observed effect was not consistent throughout the entire culture period.

The greatest differences measured were between the individual cell lines cultured and not within experimental groups of the same cell line. Growth of the individual cell lines in spinner cultures is shown in [Fig.3](#). As shown, the cell lines grew at different rates and reached different maximum viable cell concentrations in spinner culture under similar conditions. Some of the cell lines quickly dropped off in number after maximum cell numbers were reached (MH70, MH479, RH525, MH437) while the other cell lines had more prolonged survival of cells after maximum cell concentrations were reached.

Differences between the cell lines observed in spinner culture were also seen in CL 6-well cultures. Comparison of the maximum viable cell concentrations achieved in the CL 6-well to the maximum viable cell concentrations achieved in spinner flask is shown in [Fig. 4](#). The left panel represents the maximum number of viable cells per ml of culture volume reached during spinner flask culture. The right panel represents the maximum number of viable cells (mean of triplicate cell compartments) during culture of the indicated cell line in the CL 6-well. A similar pattern was



seen between spinner flask and CL 6-well cultures of the individual cell lines except for RH528. While this cell had the lowest viable cell concentrations in static culture, it reached higher values in the CL 6-well than did three of the other cell lines (RH 526, RH527, MH 437). The other cell lines followed approximately the results obtained in spinner flasks when cultured in the CL 6-well.

The concentration of antibody measured by ELISA in culture supernatants from spinner flasks and from the CL 6-well cultures harvested on day 22 are shown in Fig 5. The

highest concentration of antibody in spinner flask was obtained from MH7.0 cultures (15 ug/ml). The mean antibody concentrations measured in equal volume pooled harvests prior to day 22 and in day 22 harvests for each experimental group are shown in Table 3. An increase in antibody concentration was seen between pooled harvests prior to day 22 and the day 22 harvests for all cell lines except for RH528. This was expected as the pooled harvests included samples taken at day 7 and 9 in which maximum cell numbers were not yet achieved. The overall production pattern for the 7 different cell lines was not different between the early harvests and day 22 harvest.

Table 3

	MH7.0	MH437	MH479	RH525	RH526	RH527	RH528
Spinner	.015 mg/ml	.011 mg/ml	.012 mg/ml	< .001 mg/ml	<.001 mg/ml	.004 mg/ml	.014 mg/ml
Pooled (D7,9,13,17)	1.71 mg/ml s=0.28	1.21 mg/ml s=.29	0.26 mg/ml s=.07	.002 mg/ml s=.0001	<.001 mg/ml	.30 mg/ml s=.056	0.16 mg/ml s=.033
Day 22	2.02 mg/ml s=0.28	2.07 mg/ml s=0.16	0.31 mg/ml s=0.12	.009 mg/ml s = 0.001	< .001 mg/ml	0.49 mg/ml s=.06	0.14 mg/ml s=.06

The highest concentration of antibody was measured in supernatants harvested from the two murine hybridoma cell lines (MH7.0, MH437). The production by MH479 was less than expected predicted from the spinner culture antibody concentration of 12 ug/ml, which was similar to that obtained by MH7.0 and MH437 in spinner culture. Cell numbers obtained from the cell compartments of MH479 cultures did not correspond to antibody production. Nearly the same number of total cells were recovered from MH479 when compared to MH7.0. The two rat hybridoma cell lines RH525 and RH526 which did not produce detectable antibody in spinner culture, only one RH525 produced measurable (ug/ml) concentrations in the CL 6-well. The RH527 cell line produced only 4 ug/ml of antibody in spinner culture and produced approximately 4 fold less antibody in the CL 6-well when compared to the murine hybridomas which produced 15 and 11 ug/ml in spinner culture. The productivity of this cell line in the CL 6-well cultures was expectedly lower when compared to the murine hybridoma lines. The RH528 cell line produced 14 ug/ml in spinner flasks and yet did not produce at levels in the CL 6-well comparable to the murine hybridomas with similar productivity in spinner culture. Similar to MH479, this cell line did not produce at expected levels in CL 6-well from predictions based on static culture performance. Antibody concentrations achieved in the CL 6-well were not predicted by antibody concentrations achieved in spinner culture for all cell lines.

The antibody concentrations measured under the different experimental conditions for the separate cell lines at harvest day 22 are shown in Fig. 6. No marked or consistent differences were observed between experimental conditions. The production or concentration of antibody recovered from the CL 6-well cultures did not appear markedly influenced by the different supplementation strategies.

Summary: No consistent difference was detected in either cell growth, maximum cell concentrations or in antibody production between experimental conditions for the 7 different cell lines cultured in the CL 6-well. It was concluded that the experimental conditions were not of sufficient impact to alter culture performance of the 7 cell lines tested. Despite the range of cell types and antibody production, the addition of small amounts of FBS to nutrient medium (1%), the increase of serum in the cell compartment (15% vs 20%) or supplementation with ethanolamine was without consistent impact on culture performance. A noticeable trend in nearly all of the cultures was for maximum cell numbers to continue to increase up until approximately day 17 of culture. The different supplementation conditions did not appear to accelerate or inhibit culture growth kinetics. The delay in reaching maximum cell numbers in the CL 6-well was not explained. Dilution of conditioning factors from the initial cell inoculum may be responsible, although the stepped addition of nutrient medium did not increase cell numbers when compared to the con-



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trols and a dilution of the initial inoculum of 1.5×10^6 cells by 6 fold would appear not to be problematic, as cells were diluted further when inoculated into spinner cultures at 0.25×10^6 cells/ml. The depletion of critical growth factors from the cell compartment due to non-specific binding by the inside surfaces of the cell compartment may be responsible, although increased amounts of FBS provided to the cell compartment (15%-20%) did not accelerate growth either. Non specific protein binding would be expected to be blocked by addition of 20% FBS. It is possible that growth promoting serum factors which cross the 10,000 MWCO semipermeable membrane may be diluted by the nutrient medium. The addition of only 1% FBS to the nutrient medium may not have been sufficient to counteract the dilution. Importantly, culture conditions were sufficient to achieve expected cell numbers and indicate that supplementation of the nutrient medium with small amounts of FBS were without benefit when compared to conditions with no serum supplementation of nutrient medium. Further experiments aimed at evaluating further increased serum supplementation should address these questions.

Concentrated antibody in culture supernatant was obtained for all cell lines cultured. Even cell lines which did not produce detectable amounts of antibody in spinner flasks produced detectable antibody in the supernatant recovered from the cell compartments of the CL 6-well. Antibody was obtained at measurable amounts from supernatants of all cell lines. For the cell lines which produced reasonable amounts of antibody in static culture, a significant increase in antibody concentration was obtained. For the best producers (MH7.0, MH 437) 400 μ l of supernatant containing nearly 2mg/ml of antibody was collected in a 4 day period from a single cell compartment. A continuous batch mode of operation allowed repetitive harvest of small volumes of concentrated antibody from the cell compartment.

The cell line itself however had the greatest influence on cell growth and antibody production in the CL 6-well.

Selection for optimal cell lines may be more productive than approaches aimed at manipulating culture conditions. The small scale of the CL 6-well should provide benefits as a screening device for selecting and identifying individual clones for optimal production. Importantly, the differences in antibody production seen between spinner and CL 6-well culture indicate that for scale up in larger scale membrane based culture systems, predictions based on traditional spinner or static culture may not be accurate.

The CL 6-well device performed well as a screening tool. An experimental matrix consisting of 7 different cell lines, and 6 different experimental conditions per cell line was successfully evaluated in a short time period, with minimal consumption of medium and supplies. Importantly, triplicate measurements were obtained for each experimental condition. Further experimental conditions should be able to be readily evaluated using the CL 6-well devices and optimization of culture conditions to a particular cell line accomplished in the devices. The ability to carry out multiple replicate cultures under different experimental conditions allowed thorough investigation of the different culture conditions tested to achieve high cell density and concentrated antibody production.



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Figure 1: The mean total cell concentration for the different culture conditions of each individual cell line is shown. Mean cell counts were derived from samples removed from triplicate cell compartments during harvest. The cell concentration after harvest is shown by the vertical lines connecting the pre harvest concentration to the post harvest concentration. Cell growth is demonstrated by the return of cell concentrations by next harvest day. Total cell generation between harvest peri-

ods is indicated by the sloped lines. Rate of cell growth is represented by the slope of the connecting lines. Slower cell proliferation is seen between day 0 and day 7 than between later harvest points in the cultures. There was no consistent effect associated with a particular culture condition that was seen for numerous harvest points.

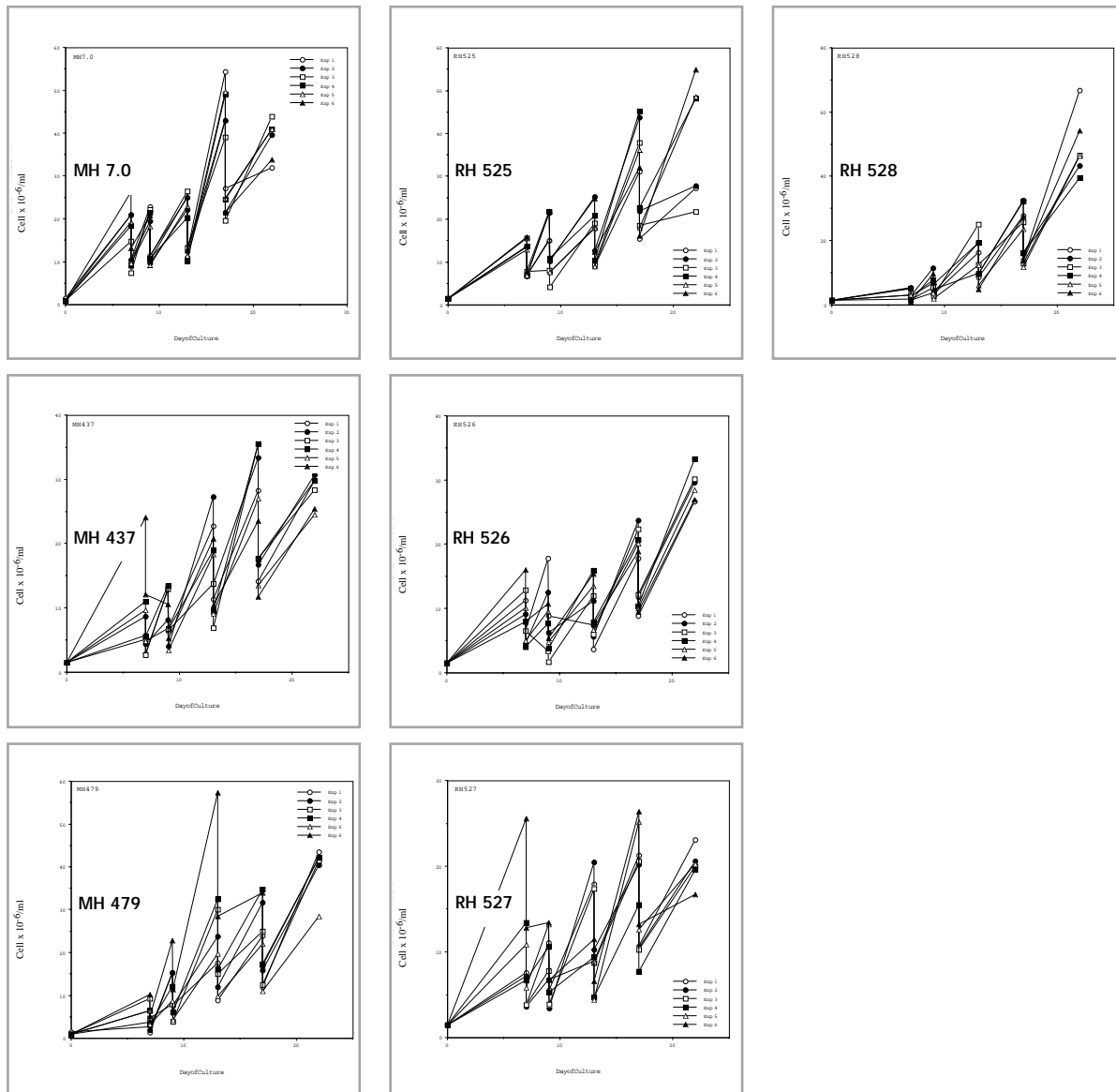




Figure 2: The mean viable cell concentrations and the standard error of the mean are shown for the different experimental groups for each cell line. The variation in the counts is shown by the standard error bars. There was no consistent effect associated with the various experimental conditions on

the viable cell concentrations measured in cell compartments on harvest day. Although marked differences were seen on a particular day, the effect was not repeated on next consecutive harvest day.

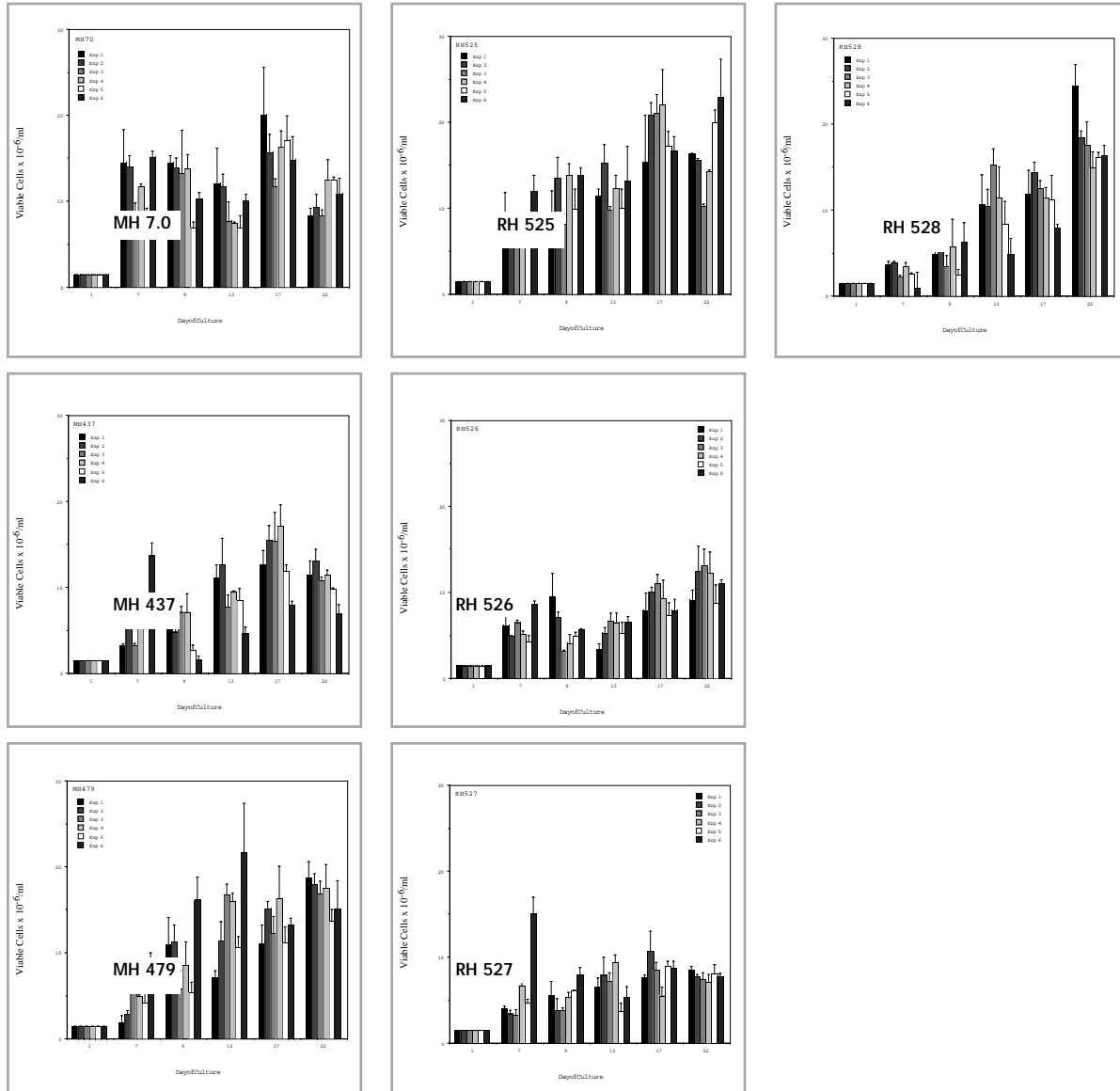




Figure 3: Cell growth in spinner culture: The number of viable cells per ml of culture is shown for the individual cell lines cultured. Differences in growth curves for the cell lines are seen. MH7.0 achieved the highest cell density, and RH528 had the lowest cell density.

Figure 4: Comparison of maximum cell concentrations achieved in spinner culture and CL 6-well. The maximum viable cell concentrations achieved in spinner culture is shown in the left panel. The maximum cell concentrations achieved in CL 6-well cultures are shown in the right panel (highest viable cell concentration over entire culture period). Correlation between cell density achieved in spinner culture and in the CL 6-well was not consistent. While a general trend was followed, exceptions were seen. RH528 while achieving the lowest viable cell concentrations in spinner culture yielded higher cell concentrations than three of the cell lines when cultured in the CL 6-well.

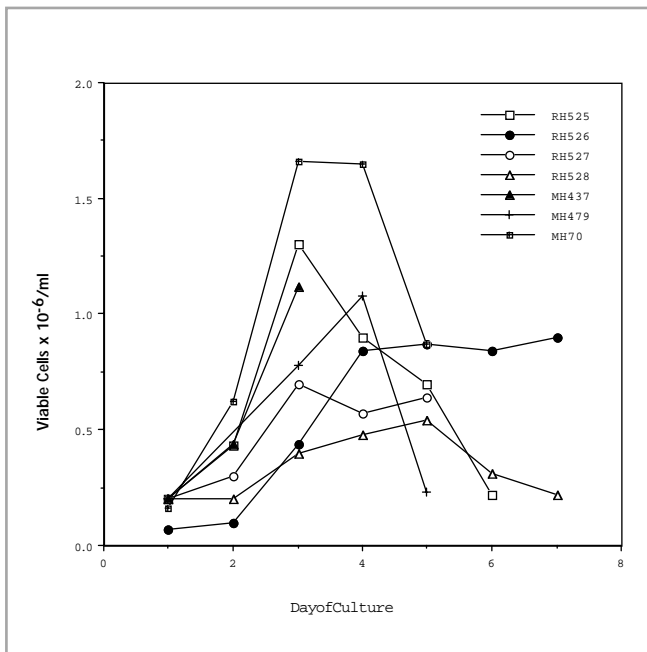


Figure 3

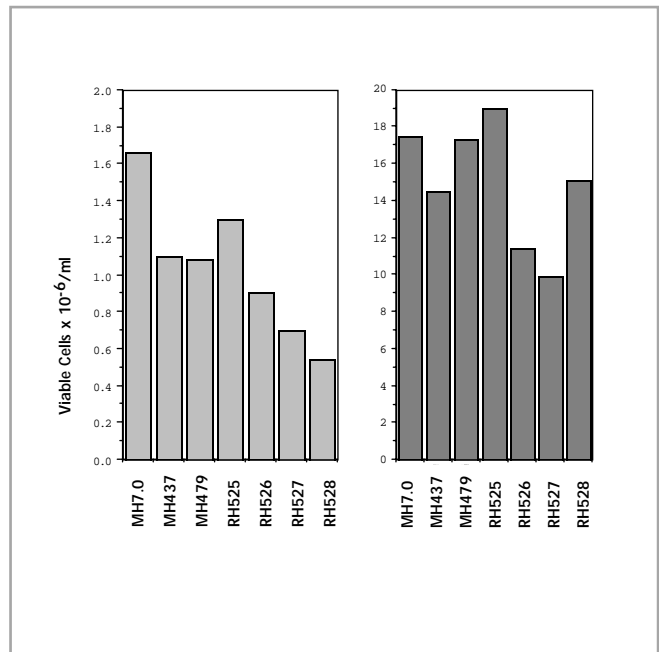


Figure 4



Figure 5: Concentration of antibody measured in spinner culture and CL 6-well (Log Scale). The maximum antibody concentration measured from the spinner cultures is represented by the light gray bars. The mean antibody concentration from the six experimental groups cultured in CL 6-well is shown by the dark bars. For RH525 and RH526 no antibody was measured in supernatant taken from the spinner cultures (<1.0 ug). Only RH525 produced measurable antibody concentrations in the CL 6-well. A lack of correlation between spinner culture production and CL 6-well production is seen for RH528, in which spinner flask antibody concentration predicted higher antibody concentrations in the CL 6-well.

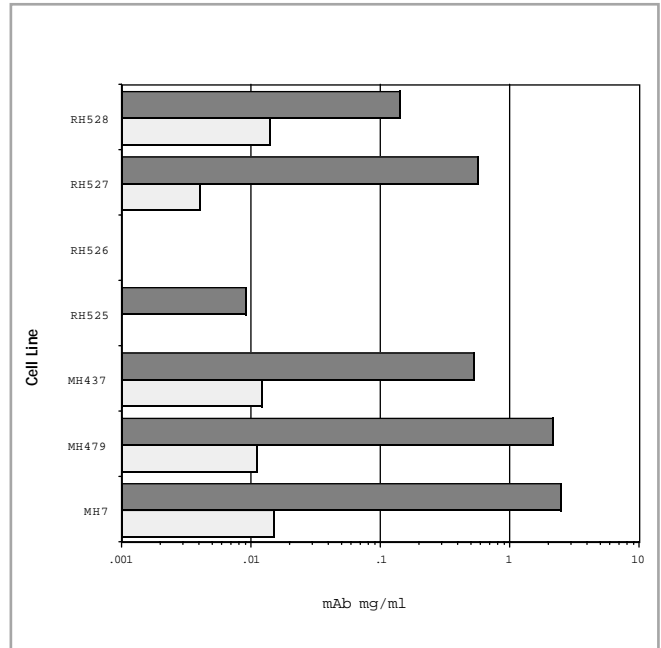


Figure 5

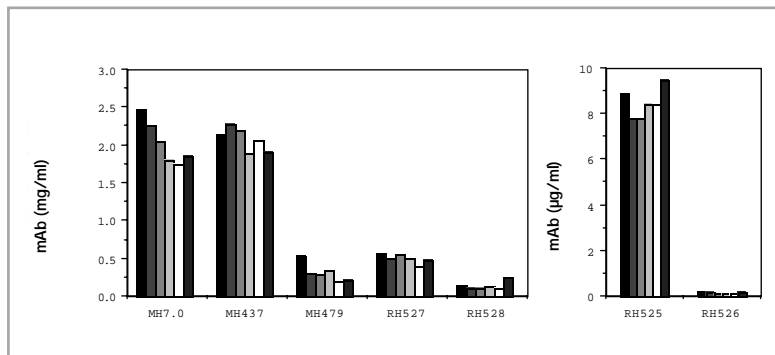


Figure 6

Figure 6: Antibody concentrations in day 22 harvest supernatants for each experimental group are shown. The left panel shows the antibody concentrations in mg/ml measured in 5 of the cell lines. The right panel shows the antibody concentrations measured in ug/ml for the remaining cell lines.