NS0 Serum-Free Culture and Applications

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Although a number of higher animal expression systems have become a regular part of the biotechnology industry, the NS0 mouse myeloma line has become one of the most popular systems in large-scale heterologous protein expression. Factors in this popularity include NS0's capacity to stably and productively incorporate foreign DNA, protein processing capability, lack of endogenous antibody production, extensive regulatory pedigree, culture as unclumped single-cell dispersions in suspension culture, robust growth and high levels of production in a variety of media, and adaptability to a variety of derivitization, selection, and production environments. Recent examples of commercially successful pharmaceutical MAbs produced in NS0 transfectoma include Centocor's Remicade and Roche's Zenapax. For reasons of regulatory compliance, cost, batch consistency, downstream processing, and materials availability, industrial applications of NS0 has moved toward one of many serum-free media platforms. Although its inherent qualities have driven its popularity, large-scale serum-free culture does pose some specific challenges. Presented here is an introduction to NS0 properties, common concerns in large-scale serum-free culture, and approaches to their resolution.

PRODUCT FOCUS: SERUM-FREE HYBRIDOMA MEDIA, HYBRIDOMA MEDIA SUPPLEMENTS

PROCESS FOCUS: LARGE-SCALE EXPRESSION, RECOMBINANT PROTEIN PRODUCTION, AND MONOCLONAL ANTIBODY PRODUCTION

WHO SHOULD READ: NS0 USERS, LARGE-SCALE PRODUCERS

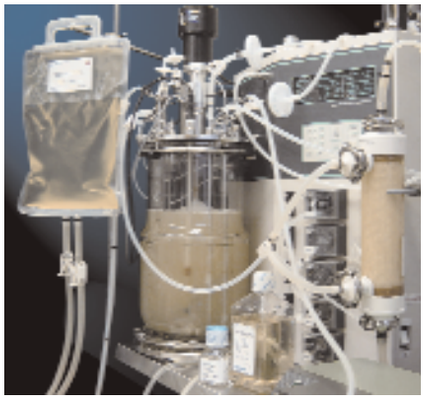
KEYWORDS: NS0, MYELOMAS, RECOMBINANT IGG, TRANSFECTOMAS, SERUM-FREE MEDIA (SFM)

LEVEL: ADVANCED

NS0 History

Lineage: Early murine myeloma lines were derived over 30 years ago and were of particular interest as stable, immortalized lines producing antibodies. As the goals for hybridoma applications advanced, requirements for myeloma lines increased. Robust cell lines were developed that were inhibited in their capacity to produce antibody and that provided an acceptable frequency of high-yield monoclonal producers upon fusion with B-lymphocytes.

NS0 is now one of many mouse myeloma cell lines available for the construction of hybridomas and transfectomas. It originated from a mineral-oil–induced plasmacytoma (MOPC-21) in a female BALB/c mouse. From the first mixed populations of cells of MOPC-21 (P3K), P3X27 was cloned, followed by 289/16 (NSI/1). Then, through 8-azaguanine selection, P3-NSI/1Ag4.1 (NS1) was isolated (1). Although all derivatives of the original tumor were high-level secretors of IgG1, this sequential cloning incrementally silenced that capacity. NS0 is a nonimmunoglobulin (IG) or Ig subunit or fragment-secreting clone derived from NS1 over 20 years ago (2). In its protracted history at many locations, it has been subcloned and otherwise selectively adapted and modified, resulting in availability of a number of distinct "NS0" lines. An example of this — and an especially strong incentive for using an NS0 line — is the relatively recent development of an NS0-based glutamine synthetase (GS) selection system. However, we here present ECACC #85110503 characteristics as nominal, referring to some of the many variant lines as appropriate (see the NS0 definition in the sidebar).



This 12-L bioreactor in perfusion mode using a 0.2-µm hollow-fiber cartridge (to culture a GS NS0 transfectoma producing human IgG in HyQ SFM4MAb and HyQ LS250) is being run in the Department of R&PD at HyClone's Logan, UT facility. (HYCLONE, LOGAN UT).

Expression Technology and Applications: Mammalian expression

technology has undergone many developments since the early days of using HAT-medium-selected hybridomas to produce mouse antibodies in ascites culture. Today, researchers can construct an NS0 transfectoma by direct cloning methods that promote high and stable expression of a fully human MAb, or other recombinant protein, under the control of multiple/inducible promoters. Production can be done in a 10k liter bioreactor using an animal-derived component and protein-free medium. Whether used in the construction of hybridomas or transfectomas, NS0 is a popular line for the production of MAbs and other recombinant proteins (**3**). The many successful selection/amplification systems reported with this line include GS (MSX), DhFr (MTX), APH (G418), HPT (hygromycin), Iapt (histidinol), HGPRT (HAT or MPA), and 3-ketosteroid reductase (cholesterol). The GS system is now the most popular with large-scale producers. Promoters/expression vectors used in producing stable and transient expression abound, and examples include pCL, pEE , pEF, pTarge, Lacswitch, and pSV. Such implementations have been used as producers of MAbs, from murine to fully human, as well as nonantibody recombinant proteins (**4, 5**). As mammalian cells, mouse myeloma lines provide all of the generic posttranslational processing steps required for authentic human product processing (**6, 7**). However, the effects of murine-specific glycan motifs, cell fusion, recombination, and overexpression levels, as well as the particular nutritional and culture environment, do affect the way and extent to which any specific process is applied to the product (**8**).

Definitions

Here are definitions of a number of abbreviations that appear in this article.

Ac-DEVD-CHO caspase-3/CPP32 inhibitor-CHO; sequence is Acetyl-Asp-Glu-Val-Asp-Asp-CHO

APH aminoglycoside phosphotransferases; Geneticin (G418) is an aminoglycoside-based antibiotic

BALB/c mouse albino (white coat with pink eyes) breeding stock obtained by Dr. Halsey J. Bagg from a mouse dealer in Ohio in 1913 (BALB = Bagg albino); the subline was incorporated into NIH main colonies in 1951

Bcl-2 B-cell lymphoma/leukemia-2, one of a large family of proteins that regulate cell death and growth.

DhFr (MTX) dihydrofolate refuctase (DhFr) and the inhibitor methotrexate (MTX)

ECACC the European Collection of Cell Cultures (www.ecacc.org.uk), established in 1984. Cell lines available include those stored by the ECACC, the European Human Cell Bank, and the Hybridoma Collection; it is also able to supply cell lines held by ATCC.

GADD153 growth arrest and DNA-damage-inducible protein; the cellular injury response gene; gene name(s) DDIT3 or CHOP or GADD153; organism source *Homo sapiens*

GS glutamine-synthetase

HSP70 the HSP70 gene encodes a major stress-inducible heat shock protein that plays an important role in protecting cells from deleterious stresses

HGRPT (HAT or MPA) hypoxanthine guanine phosphoribosyl transferase; HAT medium = hypoxanthine-aminopterin-thymidine (HAT); MPA = mycophenolic acid

IGF-1 insulinlike growth factor 1 is the primary growth promoting factor of the human growth hormone.

MOPC-21 mineral-oil–induced plasmacytoma

NS0 (from ECACC #85110503)
Cell description: Mouse myeloma
Morphology: Lymphoblast
NS0 is a subclone of NS-1, which is a nonsecreting clone of P3X63Ag8. The cells are resistant to 10 µM azaguanine and do not express IgG.
Medium: RPMI 1640 + 2-mM glutamine + 10% fetal bovine serum (FBS).
Subculture Routine: Maintain cultures 3–9 × 10,000 cells/mL; 5% CO_2; 37°C
Reference: *Methods in Enzymology* 1981;73B:3

P3-NSI/1Ag4.1 (NS1) a nonsecreting clone of P3X63AG8 extensively used in cell fusion studies and derived from P3K, the induced plasmacytoma MOPC-21; cells do not grow in HAT medium

P3X63Ag8 a line derived from the P3K cell line (a tissue culture line established from the MOPC-21 plasmacytoma); resistant to 0.1-mM 8-azaguanine and dies in HAT medium; the cells have been reported to be cholesterol auxotrophs because of a deficiency in 3-ketosteroid reductase activity.

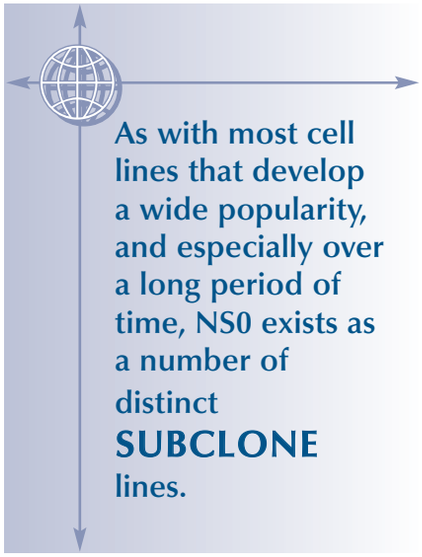
RPMI-1640 a serum dependent medium developed by Moore et al. at **R**oswell **P**ark **M**emorial **I**nstitute

Z-VAD-FMK a caspase inhibitor (FMK = fluoromethyl ketone; sequence is Z-Val-Ala-DL-Asp-fluoromethylketone)

Properties

General Characteristics: Robust performance in a variety of fusion, transfection, selection, and production approaches is a property that makes NS0 a practical candidate for product expression and construction of derivatives. Its propensity to produce stable clones of high expression efficiency means that once established, a clone can be expected to provide a consistent product over many passages (**9**). Cell-fusion efficiency was one of the early properties selected for in deriving NS0's ancestor myeloma lines. This includes its behavior in fusigens such as polyethylene glycol (PEG) and its efficiency in developing hybrids of assorted chromosomal composition. Today, its transfection efficiency in processing exogenous DNA is key. As a higher animal cell, NS0 has the potential for efficient and relatively authentic posttranslational processing. Its origin as an IgG secretor dictates its capacity for efficient production, processing, and secretion of antibody and polymeric proteins. Its current lack of endogenous Ig chain or fragment production is a great advantage in both the production and

purification of a homogeneous product and contributes to NS0's popularity (10). The fact that it maintains a nonadherent phenotype in various media and environments allows for efficient large-scale suspension culture. Protease activity in serum-free media (SFM) following cell culture is of particular importance. Both absent and



significant protease activity from NS0-derivative culture have been reported, but most reports describe no significant product degradation in application (11, 12).

Apoptosis is known to be an issue in the culture of murine myelomas and their derivative hybridomas, especially under conditions of environmental or nutritional stress. NS0 may demonstrate a particular susceptibility to apoptosis (13). A number of factors could contribute to this, including its lack of Hsp70 expression potential and variability in membrane cholesterol concentration (14). Reported inducers of the response include glucose, glutamine, phosphate, oxygen, and essential amino acid starvation; temperature, pH, and osmolality changes; and shear stress and metabolic by-product buildup. On the other hand, other reports indicate that the specific molecular onset of apoptosis in hybridomas is actually associated with cell proliferation and full metabolic activity rather than with the decline of cell viability (15, 16). GADD153 expression has been observed to be either a trigger, or at least an indicator, of NS0 apoptosis in response to environmental stress (17).

Transfection-based expression of the mitochondrial cytochrome-c active Bcl-2 has been reported to greatly reduce stress-induced apoptosis in this line, while increasing overall MAb production (18, 19). Inducible expression of the p21(CIPI) cyclin-dependent kinase inhibitor also appears to do the same (20). Some protein hydrolysates apparently provide an anti-apoptotic effect in the SFM culture of hybridomas, whereas serum and any associated growth factors do not (21). It has been observed that maintenance of ambient cholesterol levels by sequential supplementation can reduce apoptosis in suspension culture (22).

Hydrodynamic (commonly shear) force sensitivity is of concern in the suspension culture of NS0. Although every cell line exhibits its own particular milieu of characteristics in response to environmental stress, it is often convenient to generalize by referring to more or less shear "sensitive" or "tolerant" lines (23). Our observations, and those of premier commercial producers, indicate a particular issue with NS0 in this respect (12). NS0 cultures in SFM can be adapted directly to suspension culture, with appropriate supplementation; however, when moved into a bioreactor, scale-specific, apparently shear-force–induced culture impairment can occur, and especially on implementation of sparge and/or perfusion (or other cell separation) systems (24, 25).

Product secretion kinetics through the culture cycle of derivatized myelomas, including NS0, are known to be an issue. It is common to see nutrient and culture mode influences upon both the net yield and kinetics of recombinant product secretion (26, 27). A number of nutrient and culture-environment perturbations have been shown to induce increased production rates. These include reduction in either essential or nonessential nutrients (28); addition of a bolus of such nutrients (29–31); change in ambient pH (32, 33); tonicity (34); ion complement (35); CO_2 tension (36); and addition of a variety of toxicants or cytostatic agents, including those that specifically inhibit cell division or DNA replication (37, 38). Certain NS0 transfectoma lines have also been reported to produce more constitutively (less dependent on the above induction) than many hybridoma lines.

Clonal Derivatives: As with most cell lines that develop a wide popularity, and especially over a long period of time, NS0 exists as a number of distinct subclone lines. These subclones were generated by a number of ways to support particular functions, growth media, culture conditions, and selective agents. Additionally, each step of derivatization coincidentally alters other properties of the line (39, 40). Of course, the generation of hybridomas, in introducing entire lymphoid chromosomes, can introduce significant changes of phenotype. But even a transiently transfected line can exhibit distinct properties based on the clonal isolation step required to select for a productive cell concomitantly selecting for some other, accidental, property; the metabolic demands of vector expression; any required selective pressure; or the properties/processing demands of the new gene products.

In any event, significant and divergent properties have been reported in particular with individual MOPC-21 derivatives (9, 41, 42). Transgenic producers derived from the newer, commercially available GS NS0 lines are an example of this. Not only must the culture environment be modified to accommodate the GS selection system specifically, but those lines also display other significant metabolic characteristics requiring specific attention for

optimizing performance. A number of individual NS0-based producers (both hybridomas and transfectomas) have been extensively characterized in high efficiency production. Although the producers of biomedical products often maintain this information as intellectual property, published reports do provide insight into individual clonal metabolic patterns to be anticipated in any particular transgenic producer.

Note that some of the properties ascribed here to NS0 also apply to some preceding MOPC-21 derivatives. Production has been maintained through tens of passages, even in the absence of selection pressure, and the stability of derivatives through cryogenic preservation has been documented.

Special Nutritional Requirements

Cholesterol is required by all animal cells for a number of functions, including maintenance of membrane fluidity. Most cultured animal cells can produce what they require from the most elementary nutrient precursors. Nevertheless, some cells fully capable of endogenous cholesterol generation grow more efficiently with cholesterol supplementation. As the need for expression in serum-free environments developed, it was discovered that NS1 and derivatives (including NS0) had become auxotrophs for cholesterol (**43**). The biochemical step of deficiency has been identified as the demethylation of lanosterol to C-29 sterols (**44**). This means that any precursor to cholesterol above lanosterol will be ineffective as a metabolic component, and a functional supplement to the culture medium must be in the area of lathosterol to cholesterol. Most have chosen to supplement with cholesterol, although some cholesterol precursors and other sterols (stigmastanol, for example) do work (**44**). It has been observed that spontaneous cholesterol-independent revertants are quite readily generated in NS1 and NS0 (**45, 46**) as well as in P3-X27 derivatives not in the direct NS0 lineage (**47**). However, these cholesterol-independent derivatives have reduced production capacity (**12**).

Figure 1: A proprietary NS0-based transfectoma in HyQ SFM4MAb plus HyQ LS-1000. Culture was seeded at 250×10^3 cells/mL and monitored for six days without feeding or further supplementation

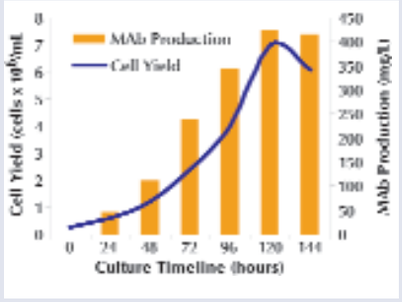
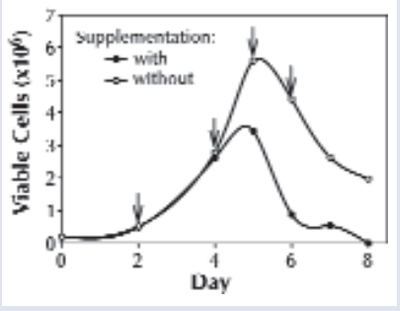


Figure 2: 125-mL shake flask culture of NS0 transfectoma initiated in HyQ SFM4MAb plus HyQ LS-1000. Culture was maintained at 150 rpm for eight days. Culture fed by the addition of HyQ LS-1000 added as noted by arrows; product level in fed culture equaled 162% of control



Glutamine supplementation is an absolute requirement for a parental NS0 line. However, the glutamine synthetase gene allowing glutamine production from glutamate and asparagine are available to NS0 from transfection (e.g., in the GS system) or fusion to other cells. Some NS0-derived hybridomas possess full glutamine synthesis function. It is reported that although NS0s do possess an endogenous GS structural gene, the generation of spontaneous glutamine-independent phenotype revertants does not readily occur, even under significant selective pressure (**48**). There are, however, reports to the contrary indicating that although the frequency of the spontaneous appearance of this phenotype is quite low (<1 in 10^7), stable glutamine-independent lines can be developed (**12**).

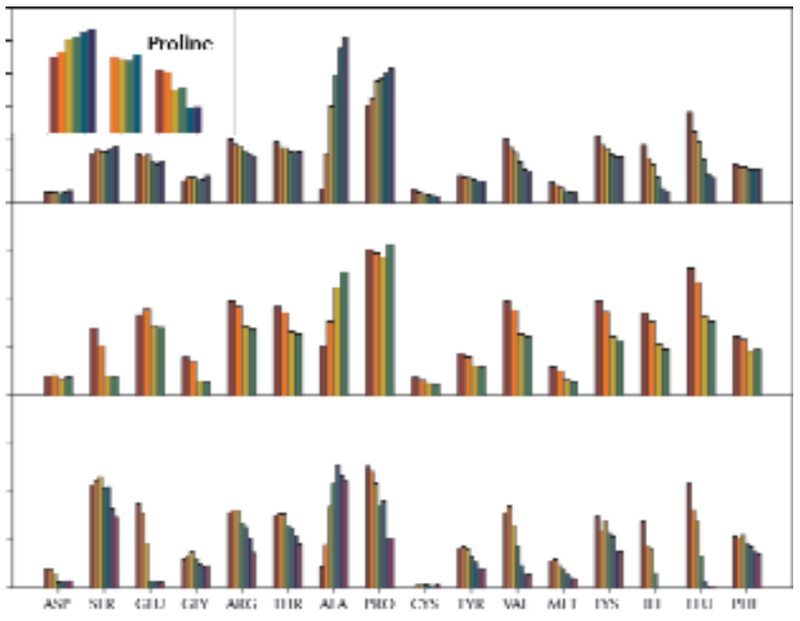
Growth Media

NS0 can be adapted to a number of commercially available cell-culture media (Figure 1). Many still use classical media, such as RPMI-1640 supplemented with animal serum. But for the reasons introduced above — and others, such as concern about induction of retroviral particle release — the number of researchers and commercial producers of recombinant proteins availing themselves of the variety of SFM available on the market today is growing. Myelomas, including NS0s, are reported to perform well in a number of custom-formulated SFMs (**49–51**). Many commercially available serum-free media have been very successfully applied, as long as they accommodate NS0's absolute requirement for significant cholesterol and glutamine. Derivatives established under serum-free conditions are observed to have acquired new media requirements or sensitivities (**12**).

General Characteristics

Serum-supplemented media of many varieties have been shown to support NS0 derivatives and good product secretion (**52, 53**). However, the issues of availability, serum cost, lot consistency, and regulatory compliance have directed many culturists to SFM formulations. Culture modes and scale appear to be more important than usual for this application. NS0s, especially in SFM culture, demonstrate a particular sensitivity to nutrient timing and the effects of impeller, sparge, and cell-separation technologies. Many of these issues have been solved, but there are some implementations of SFM, and especially protein-free culture, that remain problematic. Downstream issues have actually been reduced of late, and especially for SFM culture. Newer adsorption/affinity chemistries and resins as well as simplified purification schemes with

Figure 3: Comparative amino acid use profiles of three NS0 derivatives. NS0 transfectoma (top), NS0 hybridoma (middle), and GS-NS0 transfectoma (bottom)



increased specificity provide robust purification methods with few contraindicated raw-material components. Most commercially available SFM formulations are easily handled by existing purification schemes.

Regulatory issues and their implications are the same for NS0 derivatives as for CHO and non-NS0 myeloma. Briefly, pharmaceutical manufacturers are requiring cGMP and ISO production and strongly recommending animal-derived component-free materials in media manufacturing. Recombinant and plant-derived proteins and hydrolysates are currently acceptable, whereas for reasons of formula reproducibility, transportability, and performance constancy, the demand for chemically defined formulations is growing. Cost considerations are also the same as for other recombinant expression systems: The features of productivity, regulatory compliance, production consistency, and amenability to large-scale application allow for more complex and costly media design.

NS0-Specific Demands

Because of the diversity of their means of generation and maintenance, subclones and derivatives of NS0 complicate the identification of "NS0" media requirements. One culturist's transfectoma from a GS selection line is likely to have quite divergent media requirements from another's ATCC myeloma/mouse splenocyte hybridoma (**54**). One should keep this in mind when applying prescribed culture media formulations and protocols to any particular NS0 based line, and it is especially important when applying available "MAb Production" media, supplements, and methods, because those are often designed and tested in non-NS0 hybridoma cultures.

Autocrine and cytokine responses by myelomas and hybridomas have been explored for decades, and the various cocktails recommended are numerous and diverse. Growth-medium fractionation and add-back experimentation have shown myeloma-based lines to be totally unresponsive to, or nonsecretors of endogenous cytokines (**55**). Similar experiments have implied their existence and function in particular lines (**56**). Il-6 has been shown to be an effector in promoting certain strains derived from distant precursors of NS0 as well as hybridomas from yet other precursors (**57**). NS1 and NS0 cultures have been variously reported to be both unresponsive to, and obligate for insulin or IGF-1 (**51, 58**). Successful (albeit not optimized) performance has been observed in the culture of NS0 in quite elementary chemically defined and protein/peptide-free media, including in the absence of selenium, transferrin, or insulin/IGF-1 (**51**). Furthermore, more complex and/or commercially available chemically defined and protein-free growth media have been very successfully applied to NS0s and derivatives, achieving doubling times of 22 hours or better, cell densities >5 $\times$ 10^6/mL in batch culture, and production levels in multiples of serum containing controls (**59, 60**).

Product expression, secretion, and net production in both hybridomas and transfectomas are very dependent on nutrient supplementation, alteration, timing, and depletion as well as on other culture environment perturbations. Various culture manipulations and nutritional regimens have been reported to increase net production in hybridoma and transfectoma cultures, including those from NS0s (**32, 35, 61–66**) (Figure 2). Many represent a common theme that cultures either inhibited for (or at least introduced to a condition suboptimal for) nominal cellular proliferation, or specifically arrested at particular stages of the cell cycle, can result in higher overall product accumulation on a per cell, culture volume, or time basis (see work by AV Carvalhal in reference **4**). Others seem to be based more on the specific promotion of product secretion, such as the often published and commonly understood resistance of mouse myeloma cell IgG chain translation to inhibitors such as potassium acetate (**61**). Some of these reports are contradictory within the scope of the parameters measured, and most appear to be dependent on the derivative, medium, and/or culture configuration. This will not be specifically reviewed here, other than to observe that any of the tens of prescribed approaches (see

additional examples in General Characteristics, above) can work for a particular derivative, in a particular medium and culture configuration.

Shear protectants are required for all suspension-culture applications. Serum-supplemented media and serum-free formulations that are high in protein can provide sufficient protection for most applications. Common nonprotein protectants such as pluronic or identified fatty acids provide some protection, but NS0s can remain sensitive to aggressive sparging and cell separation techniques in bioreactor applications even at nominal levels of supplementation (**12**). Increasing pluronic to 0.2% w/v seems to provide some additional protection, but shear stress remains an issue in serum-, and especially, protein-free applications (**12, 21**).

Amino acid, vitamin, trace element, and other ion-concentration/ratio optima for various myeloma and derivatives, including NS0s, have been reported for decades (**41, 46**). General basal conditions can be established from them, as well as high-density feeding and perfusion approaches for any particular application. However, review of these formulations and direct experimentation reveal significant subclone, derivative, basal formulation, and culture-mode–induced variation in these requirements. For example, it is common to see an "NS0" reported to require supplementation of a particular amino acid in an application and discover that your culture is either not using it or is actually producing it (**59**) (Figure 3).

Figure 4: 125-mL shake flask cultures maintained at 150 rpm without feeding or further supplementation; (A) NS0 hybridoma in HyQ SFM4MAb plus HyQ LS-1000; (B) HyQ SFXMAb plus HyQ LS-1000; (C) HyQ ADCFMAb plus HyQ LS-1000; (D) RPMI 1640 plus 10% FBS

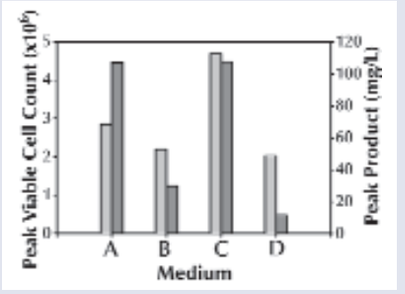
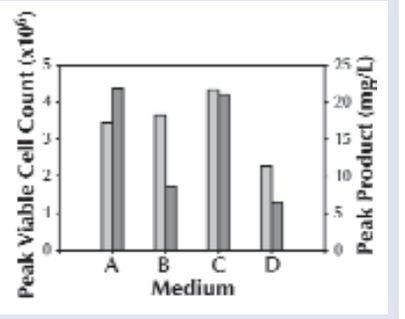


Figure 5: NS0 transfectoma in (A) HyQ SFM4MAb plus HyQ LS-1000; (B) HyQ SFXMAb plus HyQ LS-1000; (C) HyQ ADCFMAb plus HyQ LS-1000; (D) HyQ CCM-1 plus ExCyte;125-mL shake flask cultures maintained at 150 rpm without feeding or further supplementation



Apoptosis is of particular concern, especially in high-density or production culture modes. Approaches to reducing the problem include avoiding particular nutritional and environmental stresses. It appears that this issue too is greatly affected by the diversity of lines and applications used. Factors in media formulation/supplementation that can be addressed include design to avoid glucose, glutamine, and essential amino acid starvation; pH and osmolality extremes; and shear stress and by-product generation. Chemical additives shown to reduce/inhibit apoptosis may be a consideration here too. These include bongkrekic acid, cyclosporin A, pyrrolidine dithiocarbomate, N-acetylcysteine, and the caspase inhibitors Z-VAD-FMK and Ac-DEVD-CHO (**13**).

Oxygen transport is an issue in the development of any SFM. Added transferrin will normally replace the transport potential provided by serum. In protein-free formulations, a number of chelators and added iron have been shown to support most cultured cells, although many have a preference for particular complexes. Optimal NS0

performance can be obtained in this way, although some studies indicate that NS0 have a distinct phenotype in this respect, compared with other murine myeloma (**22, 50**). A relationship dependent on media composition has been observed such that a transferrin replacement chemistry that works well in one formulation, with a particular clone, may not perform well in others.

Glutamine may be available to cells in culture through absorption from the ambient medium or from the activity of glutamine synthetase on glutamate and ammonia or asparagine. To varying degrees, glutamine supplementation is a requirement for the culture of animal cells. One factor affecting the level of supplementation required is a natural variability in the capacity of cells from various tissues to express glutamine systhetase (GS). Another is the in vitro culture history of the cell line, in that ambient glutamine levels can regulate endogenous GS expression levels. In the latter case, lines that appear to have a constitutive requirement can in fact often be adapted to low/absent levels of ambient glutamine. It is of note that some SFMs identified as "w/o glutamine" in fact contain trace levels from other ingredients (e.g., hydrolysates), which while insufficient for full nutrient support, may be significant when using the GS selection system. Some glutamine dipeptides have nutritional potential in NS0 (**22**).

Lipids are a special issue in culture media supplementation because of their very limited solubility in aqueous environments. This issue is usually solved by dispersing the lipids in a microfilterable suspension through a number of approaches. The fact that NS0s require such a high concentration of a sterol lipid (2–6 mg/L of cholesterol) is a particular challenge to supplementation. Lipid supplementation issues include solubility, filterability, dispersion, physical stability, cell delivery kinetics, and container-vessel adhesion. Many technologies are available for this supplementation, including the use of vesicles, micelles, emulsions, microemulsions, carrier proteins, and carrier polymers such as cyclodextrin. Vesicles of

phospholipids in the lamellar phase can be constructed in the appropriate size and will carry significant levels of cholesterol intercalated between their acyl chains. But issues remain regarding control of the cell delivery kinetics and providing sufficient physical stability to the preparations to allow for a practical shelf life (**45**). The same issues limit the use of emulsions, which are prone to such destabilization as coalescence. Carrier proteins such as BSA work well but are both prohibited in protein-free formulations and require very high BSA concentrations at cholesterol levels required by NS0. Carrier polymers such as cyclodextrin have been found to provide a qualified solution (**67**) and can support the culture and IgG production from both NS0 hybridomas and transfectomas (Figures 4 and 5). Although cholesterol loads efficiently into certain cyclodextrins, and the resultant complex is filterable, stable, and innocuous, the nature of the association leads to instability in high dilution, determining a requirement for many supplements to be added at the time of use (**22**). A cyclodextrin-based supplement reported to be stable in high dilution is specifically proscribed by the manufacturer from supplementation in fed-batch protocols. An acceptable means of cholesterol supplementation is dependent on the medium composition. Notably, well-established high-cholesterol serum extracts have been shown in multiple labs to work well with some SFM, but not at all with others (**22**).

Some culturists are concerned about **N-glycan motifs** applied to proteins expressed in NS0s that are murine-specific (e.g., Galalpha1, 3Galbeta1, 4GlcNAc). Attempts have been made to modify N-glycan processing by manipulation of the intracellular nucleotide-sugar content. Adding identified precursors to the growth medium has yielded mixed results, affecting some residue ratios/sequences and not others (**8**).

A Proven Means of Production

The NS0 mouse myeloma line provides robust growth and production in a variety of media, and it is adaptable to a variety of manipulation, selection, and production environments. Despite identified challenges, large-scale SFM culture of NS0 derivatives is a proven means of production of antibodies and other recombinant proteins. Distinctions in the characteristics of many NS0 derivatives and clones determine the need for optimization, or at least careful selection, of the medium, culture protocol, and medium supplementation used in large-scale production.

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