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Adaptation of WM-68 Hybridoma Cell-line in Minimal Serum and Serum Free Culture Conditions

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Abstract.- WM-68 cell line has been shown to produce anti-CD48 antibody - highly specific to B cell malignancies. For their clinical usage, cultivation of WM68 cells in animal serum pose a technical and safety disadvantage. Therefore, the present study was aimed to establish an adaptation protocol for WM68 hybridoma cell line - from high to a low serum concentration and finally into a serum free culture condition. The WM68 hybridoma cell line cultured in 10% FBS exhibited excellent cell culture profiles – cell viability, density and antibody production. Similar trend was observed when WM68 cell line was cultured in 0.5% FBS. However, in 0.5% FBS, data revealed poor antibody production in the stationary phase compared to 10% FBS. Finally, WM68 cell line cultured in 0.5% FBS, was passaged and plated in a serum free condition. Unfortunately, cells didn't survive past day one and manifested obvious signs of apoptosis. Taken together, these data suggested that 10% FBS and 0.5% FBS are suitable culture conditions for the propagation of WM68 hybridoma cell line and to generate antibodies. Thus, employing additional media factors and avant-garde cell culture techniques can enhance our understanding of the WM68 cell line culture requirements in a serum free media.

Keywords: WM-68 cell line; anti CD48 antibody; Hybridoma, 0.5% FBS, serum free.

INTORDUCTION

WM-68 cell line was derived from mice after immunization with two types of cells, human-T chronic lymphocytic leukemia cells and leukemia cell line HSB2 (Henniker *et al.,* 1990; Vaughan *et al.,* 1983). These cells, cloned hybridoma (F56-3F8), secrete IgG3 - termed as WM-68. The WM-68 antibody recognizes human leukocyte cell surface antigen, called CD-48 (Henniker *et al.,* 1990). This antigen, CD48, is a glycophosphatidyl-inositol glycoprotein that is expressed by lymphocytes, monocytes and in wide range of lymphoid malignancies (Sun *et al.,* 1998). Moreover, later studies suggest that it can be removed from the cell surface by proteolytic enzymes such as papain and protease (Henniker *et al.,* 1990) and is also susceptible to the action of phosphatidyl-inositol-phospholipase C, which indicates that it is linked by phosphatidylinositol bond (Henniker *et al.,* 1990). CD48 was identified by monoclonal antibody using WM-68 and has been shown to possess the binding affinity towards B-cell malignancies including chronic lymphocytic leukemia, non-Hodgkin's lymphoma and acute leukemia (Henniker *et al.,* 1990). Literature evidences point towards a very stimulating fact that WM-68 cell line could be employed in the diagnosis, prognosis and treatment of B cell malignancies (Henniker *et al*., 1990; Smith *et al.,* 1997). However, the sustainable growth of WM68 cell line in the culture media, to acquire handsome amount of antibodies, sans animal proteins is imperative for its apt use in the clinic.

As a common practice, most hybridoma cell lines are cultured in fetal bovine serum (FBS) with conventional tissue culture media such as Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 (Darby *et al.,* 1993). However, the presence of serum in hybridoma cell culture medium adds to the fraction of immunoglobulins present in the culture media (Darby *et al.,* 1993). Literature evidence suggests that the DMEM-based-media support greater number of cells for protracted period of time compared to the RPMI-1640 based-media (Long *et al.,* 1988). Similarly, an optimally formulated DMEM-based media have been suggested as a medium of choice for the propagation and maintenance of hybridoma cell line (Long *et al.,* 1988). Though, several companies have developed

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serum free media formulations to sustain the growth of hybridoma cell lines in serum free media (Federspiel *et al.,* 1991; Tarleton and Beyer, 1991), nonetheless, specific serum free culture conditions for WM68 have not been studied yet.

In the present study, we used different concentrations of FBS in DMEM/F12 and serum free culture conditions - DMEM/F12 + ITES. The WM68 hybridoma cells exhibited viable growth with incessant production of antibody until day 6 in 10% FBS, however, slight reduction was observed when serum was reduced to 0.5% FBS. Moreover, when WM68 cells were cultured in the serum free conditions, the viability was markedly reduced leading to complete crash of the culture after day 1. These data suggest that WM68 hybridoma cells can be adapted to serum free culture conditions but to sustain long term growth and to generate sufficient antibodies, further modifications in the serum free culture conditions are necessary, thus need additional investigations.

MATERIALS AND METHODS

Procurement of chemicals and media

The chemicals employed in the study include, sodium pyruvate, sodium bicarbonate, HEPES, dimethyl sulfoxide (DMSO), ethylene-diamine-tetra-acetic acid (EDTA), phosphate buffered saline (PBS), cell dissociation solution, ferric citrate, ascorbic acid, L-glutamine and no-zyme – all purchased from Sigma Aldrich, Australia, unless otherwise specified. The versene:tryspin and ITES (Insulin 1.0 g/L; sodium selenite 0.5 mg/L, transferrin 0.55 g/L; ethanolamine 0.2 g/L) were purchased from Lonza, Switzerland. Media, Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI) and Ham`s F12, were procured from Invitrogen/Life Technologies, Australia.

Cell culture

The cells were suspended in T-175 flask (BD, Australia) at a density of 3-4 x 10^5 cells/ml in DMEM/F12 supplemented with 10% FBS. After the cells become confluent, cells were passaged in another T-175 flask. Similarly, using DMEM/F12 media culture was adapted to 0.5% FBS in a T-175 flask. Cells that get acclimatized to the 0.5% FBS culture conditions were then passaged to a serum free culture conditions upon attaining 80-90% viability. Briefly, when the percentage viability was 80-90%, the sample was centrifuged at 1000 rpm for 10 min and the cell pellet was re-suspended. Approximately, 1x10^5 cells/ml was inoculated in 0% FBS/DMEM/F12 + ITES. The media having 0% FBS/DMEM/F12+ITES were supplemented with ferric citrate solution containing ascorbic acid and L-glutamine.

Cell density and viability assay

When cells were passaged into another T-175 flask as a suspension culture, it was placed in an incubator for half an hour. After half an hour 1ml sample was taken to examine cell density and cell viability using Neubar hemocytometer and trypan blue dye, respectively. This gives us the opportunity to calculate the production of anti-body produced by each cell in a minute which is then extrapolated to an hour.

Collection of supernatant

The sample drawn at every passage was centrifuged at 1000 rpm for 10 min and supernatant was taken in an eppendrof tube to be stored at -20°C. This procedure was repeated daily until the viability dropped below 20%. At the end, the culture fluid was centrifuged at 1000 rpm for 10 minutes and supernatant was separated into a new tube and frozen.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed according to the protocol established in the lab. Briefly, one hundred micro liters of rabbit anti-mouse IgG (Dako # z0259) (1:5000) was transferred to each well of a 96-well plate and incubated overnight at 4°C or 1h at 37°C. After the incubation, the plate was washed three times with copious PBST and later blocked for 30 min in 2% skimmed milk. Sample/supernatant, collected at a specific time, and standard (100µl) was added in the assigned wells of a 96 well plate. The plate was incubated at 37°C for minimum of 45 min and washed again with PBST. After washing, 100µl of HRP (horse radish peroxidase) conjugated anti-mouse antibody (Zymed # 81-6520 Goat anti

Mouse IgG (H+L) HRP 1mg/ml) (1:2000) was added and incubated for 45 min at 37°C. Detection reagent (100µl) containing 0.06% ABTS (2,2'-Azino-bis(3-ethylbenthiazoline-6-sulphonic acid) in citrate buffer pH 4 was added to the corresponding wells. Later, the plate was left at a room temperature for a color reaction. Thereafter, the plate was read on VersaMax™ tunable microplate reader at absorbance of 405nm.

RESULTS

Improved viability and antibody production by WM68 in 10% FBS / DMEM / F12

Although presence of fetal bovine serum in hybridoma cell line culture render additional fraction of immunoglobulins in the media, studies have shown that serum considerably improves cell functions, such as viability, proliferation and antibody production (Darby *et al*., 1993). Therefore, we examined WM68 cell viability, doubling time and antibody production in 10% FBS. The presence of 10% FBS significantly improved cell viability from day 1 onward, reaching maximum at day 4, while antibody production continue to rise (D2-93%, D3-151%, D4-23% and D5-8.8%) with every passing day, despite a decrease (19%) in percentage cell viability on day 5 compared to day 1 (Table I). The population doubling time was around 20 h (Table II). Moreover, The specific production of antibodies 'q' was calculated for the exponential phase and the stationary phase showing that antibody production was higher during stationary phase ($2.5x10^{-6}$ µg/ml/cell/h) compared to exponential phase ($1.6x10^{-6}$ µg/ml/cell/h) (Fig. 1A). Interestingly, antibody production showed significant relationship between cell density and IgG concentration with corresponding days (Fig. 1B) which was further corroborated by a strong correlation (R = 0.9602) between antibody production and cell density from day 1 onward till day 6 (Fig. 1C).

Smooth adaption of WM68 cell culture in 0.5% FBS/DMEM/F12+ITES

Scientists have been carving for serum free culture conditions of cell lines/primary cells for their ardent use in the clinic. Thus, we tried reduced

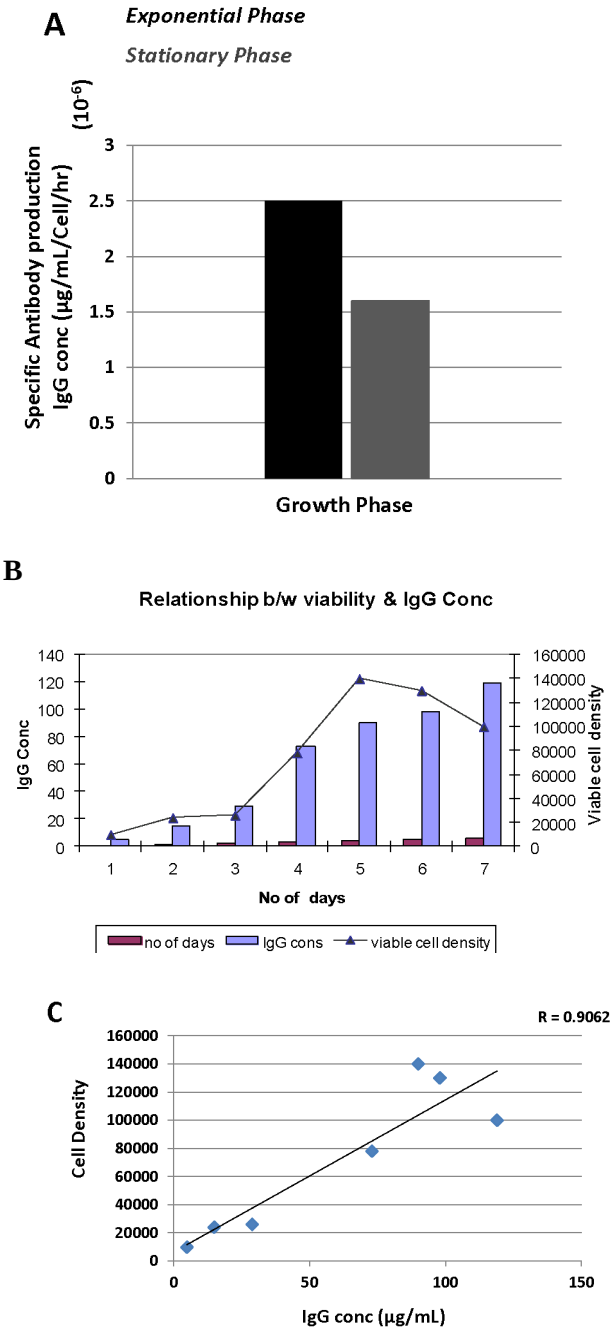

Fig. 1. WM68 exhibited healthy cell culture profiles; cell viability, density and antibody production. A) Specific antibody production during exponential and stationary phases. B) Relationship between the number of days, cell viability, density and antibody production. C) Correlation between cell density and antibody production.

Table I.- Effect of serum and serum free culture on the cell density, percentage viability and IgG production by WM68 hybridoma cell line in different culture conditions.

No. of days	% viability			Viable cell density (cells/ml)			IgG concentration (µg /ml)		
	10%	0.5%	0%	10%	0.5%	0%	10%	0.5%	0%
0	73	96	92	9.8×10^4	6.25×10^4	5.75×10^4	5	28	0
1	84	82.0	75	2.4×10^5	1.22×10^5	6.00×10^5	15	30	0
2	85	82.0	0	2.6×10^5	2.4×10^5	0	29	53	0
3	85	87.2	0	7.8×10^5	4.2×10^5	0	73	62	0
4	86	88.0	0	1.4×10^6	7.9×10^5	0	90	72	0
5	68	75	0	1.3×10^6	5.15×10^5	0	98	78	0
6	63	0	0	1.0×10^6	0	0	119	0	0

Table II.- Population doubling time and specific antibody production by WM68 in 10% and 0.5% FBS

Media condition	Doubling time 'h'	Stationary Phase 'q' (µg/cell/h)	'Exponential Phase 'q' (µg/cell/h)
10% FBS	20	1.6×10^{-6}	2.5×10^{-6}
0.5% FBS	25	1.5×10^{-6}	0.38×10^{-6}

amount of serum concentration to finally steer into the serum free culture conditions. A moderate increase in population doubling time (25 h) was observed compared to 10% FBS culture (PDT; 20h) (Table II), however, the reduction in serum concentration was 95% compared to the increase in population doubling time (25%) (Table II). Similarly, maximum cell viability (88%) was observed on day 4 which was decreased (15%) to 75% on day 5 (Table I). However, antibody production at specific day, corresponding particular cell density or cell number was higher when cultured in 0.5% FBS compared to 10% FBS, *e.g.*, on day 4 (10%FBS), 1.4×10^6 cells produce around 90 µg/ml of antibody (6.4×10^{-5} µg/ml/cell), while at the same day in 0.5% FBS - 7.9×10^5 cells produced 72µg/ml of antibody (9.1×10^{-5} µg/ml/ cell) (Tables I, II). Likewise, cell viability and IgG concentration showed significant relationship until day 5 of the culture (Fig. 2B) and was further substantiated by a sturdy correlation (R = 0.888) between IgG concentration and cell density (Fig. 2C). However, marked reduction in antibody production was observed in the stationary phase of WM68 in 0.5% FBS compared to the stationary phase of WM68 in 10% FBS (Fig. 1A, 2A).

WM68 adoption in serum free culture conditions; a spontaneous culture crash

Animal serum is frequently used in tissue culture experiments as a source of nutrients but contain many ill-defined factors thus posing a technical disadvantage of its inclusion. Fetal bovine serum (FBS) comprises of several imprecise factors, whilst, vital for cell proliferation, attachment and differentiation (Brunner *et al.*, 2010). Thus we aimed at a smooth transition from 0.5%FBS to a serum free culture for our WM68 cell line. The cells were passaged from 0.5% FBS medium to protein-free medium supplemented by ferric citrate solution when the viability was highest (88%) on day 4 (Table I).

Initially, culture showed impressive viability of 92% upon plating (Table I), however, on day 1 a sudden decline in cell viability was observed, showing spontaneous crash of the culture (Table I), and signs of apoptotic morphology – cells become flat with increased number of vacuoles. Moreover, samples were not viable for antibody concentration tests.

DISCUSSION

The cell culture model systems are increasingly employed and have become indispensible tool in basic bio-medical research (Brunner *et al.*, 2010; McKeehan *et al.*, 1990). Similarly, propagation and expansion of human cells for tissue engineering, regenerative approaches employing stem cells and cell based therapies have gained much awaited attention (Bianco and Robey, 2001; Eridani *et al.*, 2004). However, most of the

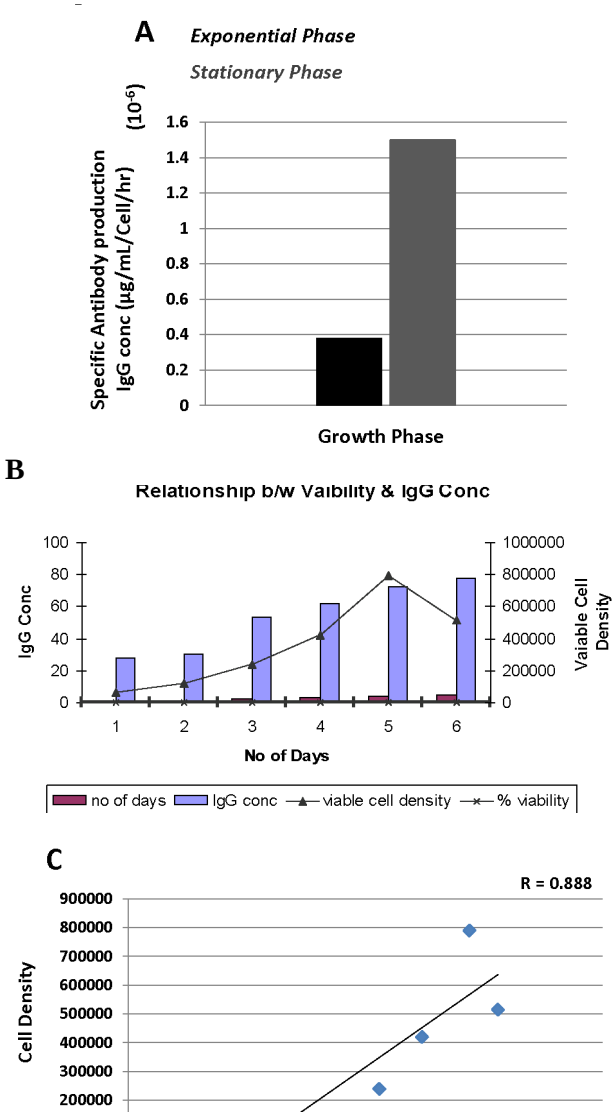

Fig. 2. Smooth adaption of WM68 cell line in 0.5% FBS culture conditions. A) Specific antibody production during exponential and stationary phase. **B)** Relationship between the number of days, cell viability, density and antibody production. **C)** Correlation between cell density and antibody production.

in vitro culture approaches include the use of animal serum, most commonly FBS, to provide basic nutrients and factors for appropriate proliferation, attachment and other cell functions (Brunner *et al.*, 2010; Ahmad *et al.*, 2012). More recently, advancement in hybridoma and stem cell research lead to the development of more innovative and stringent cell culture modus operandi to be employed in antibody production, stem cell based therapies and tissue engineering modalities such as, utilization of proteins and growth factors of human origin for autologous cell expansion and re-implantation (Czyz *et al.*, 2003; Eridani *et al.*, 2004; Risbud and Sittinger, 2002). Furthermore, removal of animal sera from hybridoma media renders numerous technical and safety benefits, such as, simple purification of antibodies from the culture supernatants, reduce risk of contamination by microorganisms and reduction in reagent cost (Brunner *et al.*, 2010). In this report, we examined the adaptation of WM68 cell line from 10% FBS to 0.5% FBS and finally into a serum free culture conditions. Data showed that WM68 gave pertinent responses in terms of % viability, cell density and antibody production in 10% FBS and was smoothly adapted to a more than 100% reduction in serum concentration that is 0.5% FBS. However, transition from 0.5% FBS to serum free culture conditions was not sustainable for more than a day and the culture crashed suddenly after one day.

WM-68 was grown in two different serum concentrations and was subjected to a process of adaptation so that the cell line could be cultured in serum free (0% FBS) medium. Data showed that, upon plating, at day zero in 10% FBS, the cells exhibited reduced viability (73%), cell density ($1x10^4$) and secreting minimal amount of antibodies (5µg/mL). This could be explained by the presence of most of the cells in the lag phase – adapting to the *in vitro* culture conditions and also because of cell density, since cells plated at higher density have better cell to cell communication that helps in apt acclimatization to the culture conditions and in appropriate cell functions (Lee *et al.*, 1989a,b). However, upon entering into an exponential phase, the cell density increased considerably from ~10^5 to ~10^6 and the production of antibody was also increased from 15µg/ml on day 2 to 90µg/ml on day 4, respectively, suggesting that antibody production is related to metabolic activity of the cells (MacMichael, 1989). These responses by WM68 in 10% FBS were expected and many labs have reported the similar effects of serum on hybridoma cell viability, density and antibody production (Lee *et al.*, 1989a,b; MacMichael, 1989). Interestingly,

even though the cell density and viability were decreased in the stationary phase the subsequent production of antibody did not decrease, rather the cells produced significant amount of monoclonal antibody (119µg/ml) – the highest. The likely reason would be that all the viable cells, despite reduced cell viability and density, are producing antibodies and might be in G2+M phase of cell cycle, since, the secretion of antibody in G2+M phase was reported almost 3 and 5 times higher than G1 and S phase, respectively (Kromenaker and Srienc, 1994). Another plausible explanation could be that in exponential phase the cells undergo mitosis, utilizing more of the nutrients from the medium and most of the acquired nutrients are consumed in the synthesis of proteins for the sustenance of their life, consequently the cells do not produce much antibodies (Hayter *et al.,* 1992). This was further confirmed when hybridoma's were transitioned from 10% FBS to 0.5% FBS culture conditions. Despite reduction in the serum (0.5%) and the cell density, the antibody production was comparable or even more than 10% FBS culture, and corresponds to the number of cells producing specific amount of antibody. For example, at day 4, having maximum viability, in 10% FBS, $1.4x10^{6}$ cells produce 90µg/ml of the antibody; $6.4x10^{-5}$ µg/ml/cell, while in 0.5% FBS at the same day $7.9x10^{5}$ cells produce 72 µg/ml of the antibody; $9.1x10^{-5}$ µg/ml/cell, almost 42% higher based on the number of cells producing the antibodies - suggesting that the proliferation and antibody production are independent of each other (Hayter *et al.,* 1992). However, more strikingly, the specific antibody production in 0.5% FBS was lower compared to 10% FBS in the stationary phase, pointing towards the lack of growth factors and nutrients, in 0.5% FBS, vital for sustaining the functions of the cells during the stationary phase.

Nevertheless the daunting task was to make a smooth transition in a serum free culture conditions. Our data suggested that WM68 cell line could not survive after day one, with complete crash of the culture and manifest signs of apoptosis, such as flat morphology and increased number of vacuoles. Several studies have demonstrated that hybridoma cell line can be cultured in serum free conditions and various labs have tried different approaches and supplementary reagents, such as, combining several media and culturing hybridoma in 1%FBS which was later adapted to serum free culture conditions (Schneider, 1989), addition of insulin, transferrin, ethanolamine and selenium to the media sans serum (Kovar, 1988; Murakami *et al.,* 1982), others have tried ITES to culture hybridoma in serum free media (Takazawa *et al*., 1988). However, the culture conditions and supplemented growth factors and nutrients vary from lab to lab, so as the results coming from each lab and the hybridoma cell lines employed for the studies. We passaged the WM68 cell line culture from 0.5% FBS having 87% cell viability, into a serum free culture conditions; supplemented with ITES, ferric citrate and ascorbic acid. Ferric citrate and ascorbic acid has been shown to promote cell growth and differentiation (Kovar, 1988; Ulrich-Merzenich *et al.,* 2007). Despite, plating highly viable cells and using already tested factors supplemented with growth promoting factors, such as ferric citrate and ascorbic acid our culture didn't survive past day one. Seemingly, WM68 cell line requirement might be different from hybridoma cell lines that have been cultured in serum free conditions employing ITES only. However, some have tried a different approach such as generation of serum free fusions, although fewer, to further cultivate the hybridoma in serum free conditions (Liu *et al.,* 1998). However, it is also plausible to expect the cell type specific responses regarding the generation of fusions, when executed in serum free or reduced serum conditions.

In conclusion, our data suggested that WM68 hybridoma cell line can be cultured in 10%FBS without compromising cell viability, density and antibody production. Similarly, a smooth transition of WM68 hybridoma cell line, cultured in 10%FBS, was observed when plated into 0.5% FBS culture conditions. However, despite excellent cell viability in 0.5% FBS, when cells were plated into serum free media supplemented with ITES, ferric citrate and ascorbic acid, known for their growth promoting effects, the culture didn't survive past day one, with obvious signs of apoptosis. It is plausible that changes in the pH might be one factor contributing towards the culture crash, however, effect of other factors like, reduced glucose and glutamine concentration in the media or other unknown

factors, present in the serum, but not in the serum free conditions, might affect the cell survival and function.

Thus, further studies are imperative to standardize serum free culture conditions for WM68 cell line, that may also require additional growth factors and nutrients along with manipulation of the culture conditions such as, mixing media in different ratios, adaption of culture to the decreasing amount of serum, generation of fusions in serum free conditions and adding survival genes during clone formation.

Conflict of interest

All authors have no conflict of interests.

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