Hybridokine

REF Hykine 12.5 Product Info Sheet RUO *For Research Use Only*

Highly effective enhancing factor for hybridoma development

1. Introduction

One of the key issues in hybridoma technology is cell cloning and culturing. Normally cloning is performed via limiting dilution shortly after fusion. Only in this way establishment of relevant clonal hybridomas is ensured. To sustain growth of hybridomas, the normally used culture media have to be supplemented with additional growth factors.

A lot of research has been devoted to optimize media and their supplements (i.e. growth factors) for cloning procedures. In addition, to facilitate down-stream processing of monoclonal antibodies (e.g. purification), much effort has been put in minimizing the content of irrelevant proteins (e.g. irrelevant Ig IS) in hybridoma growth media.

Hybridokine, cloning efficiency enhancing factor, which meets the demands of hybridoma propagation during limiting dilution protocols.

2. Hybridokine: Cloning efficiency enhancing factor

Hybridokine has been developed in order to increase the yield of newly developed hybridoma cells after (re)cloning.

Frequently feeder layers are used for initial seeding and cloning of hybridoma cells, but their cultivation and preparation is a very time-consuming process. Moreover, production of feeder layers requires animal facilities and irradiation equipment and their activity may vary considerably.

Hybridokine can replace feeder cells completely to Support clonal hybridoma growth. **Hybridokine** secreted by a clonally selected human Ewing Sarcoma-derived cell line. This cell line is cultured under controlled conditions using FBS with USDA certificates. As a lyophilized product **Hybridokine** is very stable and can be made ready to use simply by addition of demineralized water.

In order to illustrate the effects of **Hybridokine** both on cloning efficiency and on cell growth these effects are studied in a number of experiments, described below. In these experiments **Hybridokine** was compared to the growth factor Interleukin-6.

The superior effect of **Hybridokine** is clearly demonstrated.

3. Does Hybridokine contain Interleukin-6

In many hybridoma growth stimulating products, the active compound is known to be Interleukin-6. Using an Interleukin-6 ELISA (Medgenix, Belgium), **Hybridokine** was analyzed for its Interleukin-6 content. **Hybridokine** proved to contain 2,000 (± 5%) units Interleukin-6 per ml.

In order to demonstrate the presence and activity of other stimulating factors than Interleukin-6 in **Hybridokine**, the preparation was depleted from Interleukin-6 by the addition of a 500-fold molar excess of monoclonal antibodies against Interleukin-6 and subsequent removal of the antibody/Interleukin-6 complex by Protein-A incubation. This resulted in an 80% decrease of the Interleukin-6 content of Hybridokine™. Interleukin- 6 depleted **Hybridokine** was used for comparison in a number of experiments.

As shown in the experiments presented on the next pages, the stimulatory effect of **Hybridokine** on hybridoma cloning efficiency and on hybridoma growth rate is not solely due to Interleukin-6 activity.

4. Effect of Hybridokine and Interleukin-6 on cloning efficiency of hybridoma cell line MB-3

In a typical cloning protocol, developing hybridomas are diluted in micro titer plates to a cell concentration of one cell per well. These conditions are extremely critical for cell growth but necessary for obtaining clonal hybridomas. Based on such methods, we have demonstrated the stimulating effect of **Hybridokine** using a concentration of 20 cells per well.

In all experiments MB-3, a hybridoma cell line, has been used. This cell line was derived from a fusion between spleen cells of immunized Balb/c mice and the mouse myeloma cell line P3-X63-Ag8 (ref. 1). Comparable results were obtained with a variety of other hybridoma cell lines.

Protocol

MB-3 cells were grown in an RPMI-1640 medium supplemented with **Hybridokine** in a final concentration of 2.5% (v/v). Cells were harvested by centrifugation and were washed three times in a medium without **Hybridokine** and viable cells were selected using a FIuorescence Activated Cell Sorter (FACS). Subsequently, 20 viable cells per well were sorted in 96 wells micro titer plates under sterile conditions, after which liquid softagarose was added to the wells. The micro titer plates were incubated for 30 minutes at 4 °C. After congelation 100 µl of growth medium containing increasing concentrations of **Hybridokine** [0 - 8% (v/v)] or recombinant Interleukin-6 [0 - 8% (v/v)] was added to the plates. Since **Hybridokine** contains the same amount of Interleukin-6, direct comparison can be made with an Interleukin-6 stock solution containing 2,000 units recombinant Interleukin-6 per ml.
Alternatively, **Hybridokine** was depleted from Interleukin-6 as described above. The resulting material was also added to micro titer plates, comparable to the method described above. The micro titer plates were incubated at 37 °C in a CO2 incubator.

After 7 -10 days of incubation the number of evolving clones per well was counted (16 wells per assay) and averaged. Cloning efficiency is defined as the ratio between the number of colonies evolved per well (0 to 20 colonies) to the number of cells seeded per well (20).

Results and conclusions

As shown in figure 1, **Hybridokine** strongly enhances the cloning efficiency of MB-3 cells. Removal of 80% of the Interleukin-6 content of **Hybridokine** did not significantly effect the stimulating effect of **Hybridokine** on cloning efficiency.
Results from comparable experiments with Interleukin-6 showed, that recombinant Interleukin-6 could not mimic the effect of **Hybridokine** on cloning efficiency, indicating that in **Hybridokine** other components than Interleukin-6 are responsible for the stimulatory effect on cloning efficiency (see figure 2).

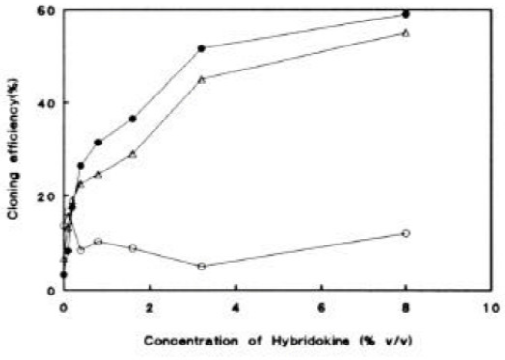

Figure 1: Cloning efficiency
Effect of increasing concentrations of Hybridokine (?) and Hybridokine treated with a 500-moIarr excess of monoclonal antibodies anti-Interleukin-6 (?) on the cloning efficiency of MB-3 cells. As a control, the effect of increasing concentrations of RPMI-medium 1640 with 8% (v/v) FBS (?) is given. Cloning efficiency has been determined as described.

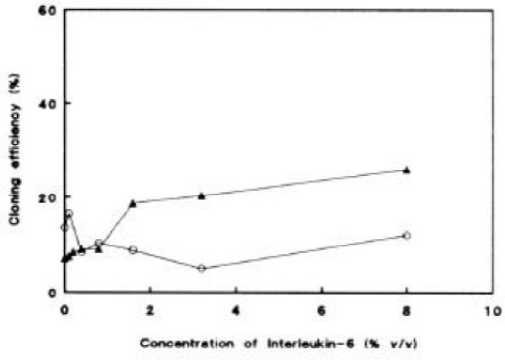

Figure 2: Cloning efficiency
Effect of increasing concentrations of a stock solution of recombinant interleukin-6 (2000 U/ml) (?) on the cloning efficiency of MB-3 cells. As a control, the effect of increasing concentrations of RPMI-medium 1640 with 8% (v/v) FBS (?) is given. Cloning efficiency has been determined as described.

It is concluded that **Hybridokine** has a stimulatory effect on hybridoma cloning efficiency that is caused by factors different from Interleukin-6.

5. Effect of Hybridokine and recombinant Interleukin-6 on hybridoma

Like many other factors that have been described to stimulate hybridoma cell-culturing Hybridokine™ increases the growth rate of hybridomas. This effect has been compared to the growth stimulation by recombinant Interleukin-6.

Determination of the growth rate of MB-3 cells
To determine the growth rate of hybridomas, a 3-[4'5'-dimethylthiazol-2-yl]-2,4-diphenyltetrazolium bromide (MTT) assay was used (ref. 2). To that end, MB-3 cells were grown in a RPMI-1640 medium supplemented with **Hybridokine** at a final concentration of 2.5% (v/v). Cells were harvested and washed in a medium without **Hybridokine**.
MB-3 cells were harvested and washed with medium without **Hybridokine** and analyzed for viability using a Fluorescence Activated Cell Sorter (FACS). Subsequently, 2,000 viable cells per well were sorted in the microtiter plates under sterile conditions.

Microtiter plates (96-wells) were filled with 200 µl of medium containing increasing concentrations of **Hybridokine**, a solution containing 2,000 units/mI recombinant Interleukin-6 or **Hybridokine** depleted of Interleukin- 6 as described above [0 - 8% (v/v)]. The microtiter plates were incubated at 37 °C in a CO2 incubator.

After 5-6 days of incubation the growth rate was determined by measuring the formation of reduced MTT (formazan).

MTT was added to the wells to a final concentration of 0.4 mg/ml and the micro titer plates were incubated in the CO2 incubator at 37 °C for 4 hours. The formation of formazan was stopped by the addition of a solution of 10% (w/v) sodium dodecylsulphate in 50% dimethyl formamide, pH 4.7. The microtiter plates were incubated overnight in a CO2 incubator at 37 °C. Subsequently, the formation of formazan was determined by measuring the absorbency at 580 nm.

Results and conclusions

Treatment of **Hybridokine** with excess of monoclonal antibodies anti-Interleukin-6 reduced the Interleukin-6 content to about 20%, but did hardly alter the effect on growth rate of MB-3 cells (see figure 2).

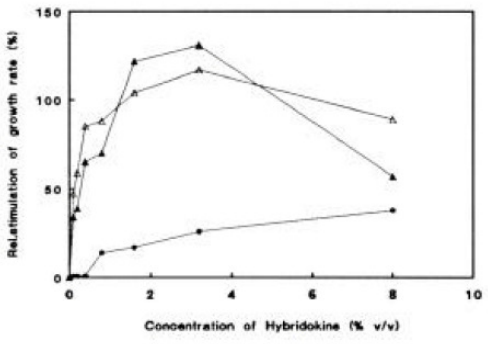

Figure 3: Growth rate
Effect of increasing concentrations of Hybridokine (?) and Hybridokine treated with a 500-molar excess of monoclonal antibody anti-Interleukin-6 (?) on the growth rate of MB-3 cells. As a control, the effect of increasing concentrations of RPMI-medium 1640 with 8% (v/v) FBS (?) is given. The relative stimulation of growth rate has been determined.

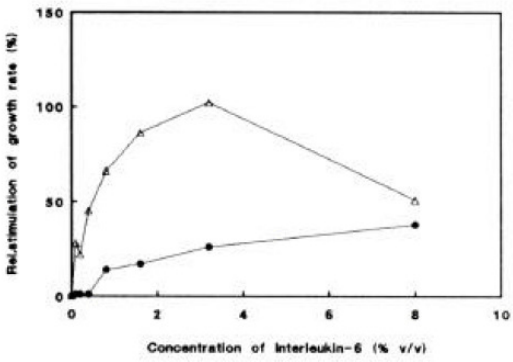

Figure 4: Growth rate
Effect of increasing concentrations of recombinant Interleukin- 6 (2,000 units/mI) (?) on the growth rate of MB3 cells. As a control, the effect of increasing concentrations of RPMImedium 1640 with 8% (v/v) FBS (?) is given. Growth rate has been determined as described. The relative stimulation of growth rate has been determined.

Recombinant Interleukin-6 added to the growth medium to a final concentration comparable to **Hybridokine** has a similar effect on growth rate as **Hybridokine** (> 85%) (see figures 3 and 4). High concentrations of rInterleukin-6 (> 60 units Interleukin-6/ml) added to the medium to reduce the stimulatory effect on growth rate of MB-3 cells. It is concluded that the stimulatory effect of Hybridokine™ on the growth rate of MB-3 cells is comparable to but not caused by Interleukin-6 only.

6. General conclusions

Hybridokine effectively enhances the cloning and growth of hybridoma cells. **Hybridokine** can replace feeder cells completely and is thus a very useful factor to supplement media for developing, cloning and culturing of hybridomas.

Hybridokine contains a significant concentration of Interleukin-6. However, stimulatory effect of **Hybridokine** on the growth rate of MB-3 cells results from other factors in addition to Interleukin-6. In general it can be concluded that **Hybridokine** is a very effective cloning and growth-enhancing factor.

7. Literature references

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