

Heat Inactivation— Are You Wasting Your Time?

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An issue that should be of interest to most cell culturists is the heat inactivation of serum products. It would be inconceivable to purchase thousands of dollars worth of expensive growth factors, vitamins, amino acids, etc., and needlessly expose them to temperatures exceeding 56°C for at least 30 minutes. Nevertheless, this is exactly what takes place in many cell culture laboratories. Many cell culturists routinely heat inactivate serum products without considering how this extreme treatment affects growth factors, vitamins, amino acids, etc. One of the most frequent inquiries that we receive on our Technical Hotline is whether to heat inactivate serum. To address this concern and further bring cell culture from an art to a science, we investigated the effects of heat inactivation on fetal bovine serum (FBS).

According to some estimates, at least 70 percent of all customers who heat inactivate serum do so simply because it is included in their protocols or it has always been accepted as necessary. Recommended temperatures in the protocols range from 45°C to 62°C and times range from 15 minutes to 60 minutes. The most common procedure involves subjecting the serum to 56°C for 30 minutes. Many of these protocols originated before the 1970s and have not been questioned. As a result of improvements in the collection, processing, and understanding of serum, many of the original reasons for heat inactivating serum are no longer valid. Only a small percentage of customer who heat inactivate their serum have actually performed experiments to verify the efficacy of the process and determined whether heat inactivation is indeed necessary. Heat inactivation was once deemed necessary to destroy heat labile components, such as complement. However, it is apparently unnecessary to heat inactivate FBS to inactivate complement. Triglia and Linscott¹ assayed nine complement components, conglutinin and C3b inactivator in commercial FBS. The FBS contained approximately 1-3 percent of adult levels of conglutinin, C1 and C6, and 5-50 percent of adult levels of the remaining components except C3, which was undetectable. We have experienced similar results in our complement fixation assays; in 10 samples of FBS from different production lots, there was no hemolysis even in undiluted serum. Additionally, serum samples sent to an independent laboratory for complement fixation assays yielded no red cell lysis in the lowest serum dilution tested (1:4). Furthermore, cell culture laboratories often warm their serum-containing media to 37°C prior to use, which in itself would inactivate heat-labile complement factors.

In addition to complement inactivation, heat inactivation was believed to inactivate adventitious microbial contaminants such as mycoplasmas. Years ago, serum was filtered through 0.45 µm pore-size rated filters and mycoplasmas were occasionally isolated from serum and media products. In response to this concern, we were the first serum supplier to filter serum through three consecutive 0.1 µm pore-size rated filters. The continual improvement and validation of modern filtration systems has eliminated the need to rely on heat to inactivate mycoplasmas.

There are few published reports concerning the effects of heat inactivation on serum products, but our findings and customer comments indicate that heat inactivation is not necessary for most cell culture applications.

Pinyopummintr et al.² determined that it is not necessary to heat inactivate serum with high embryotropic properties and that eliminating heat inactivation does not affect bovine embryo development in vitro. It has been reported that the heat inactivation of FBS and bovine calf serum reduces their capacity to promote cell attachment.³ In tests for cell attachment activity with SV-BHK, BALB-3T3, CV-1, and FS-4 cells, heat inactivation had less effect on FBS than on bovine calf serum. Following cell attachment, heat inactivation did not seem to affect cell growth. It has also been reported that heat inactivation is not necessary for serum used with insect cell cultures and in studies of baculovirus expression.⁴

We investigated the effects of heat inactivation on FBS and its ability to support cell growth on a variety of cell lines using the most widely accepted protocol, which is described in Figure 1.

Figure 1. Protocol for heat inactivation of serum products.

1. Thaw the serum and mix the contents of the bottle thoroughly.
2. While the serum is thawing, prepare a control bottle containing water. The control bottle should be stored along with the serum bottles to ensure identical beginning temperatures. This control bottle will be used to monitor the temperature and should be identical to the serum bottle (eg., PETG).
3. Place the bottle of serum and the control bottle in a 56°C water-bath containing sufficient water to immerse the bottle above the serum level. Suspend a thermometer or thermocouple in the water bottle. The thermometer should not touch the sides or bottom of the bottle.
4. Swirl the bottles every 10 minutes. for FBS and BCS and 5 minutes. for equine serum to ensure uniform heating of the serum.
5. Monitor the temperature of the control bottle closely and begin timing as the temperature reaches 56°C.
6. After 30 min. at 56°C, immediately cool the bottles of serum in an ice bath.

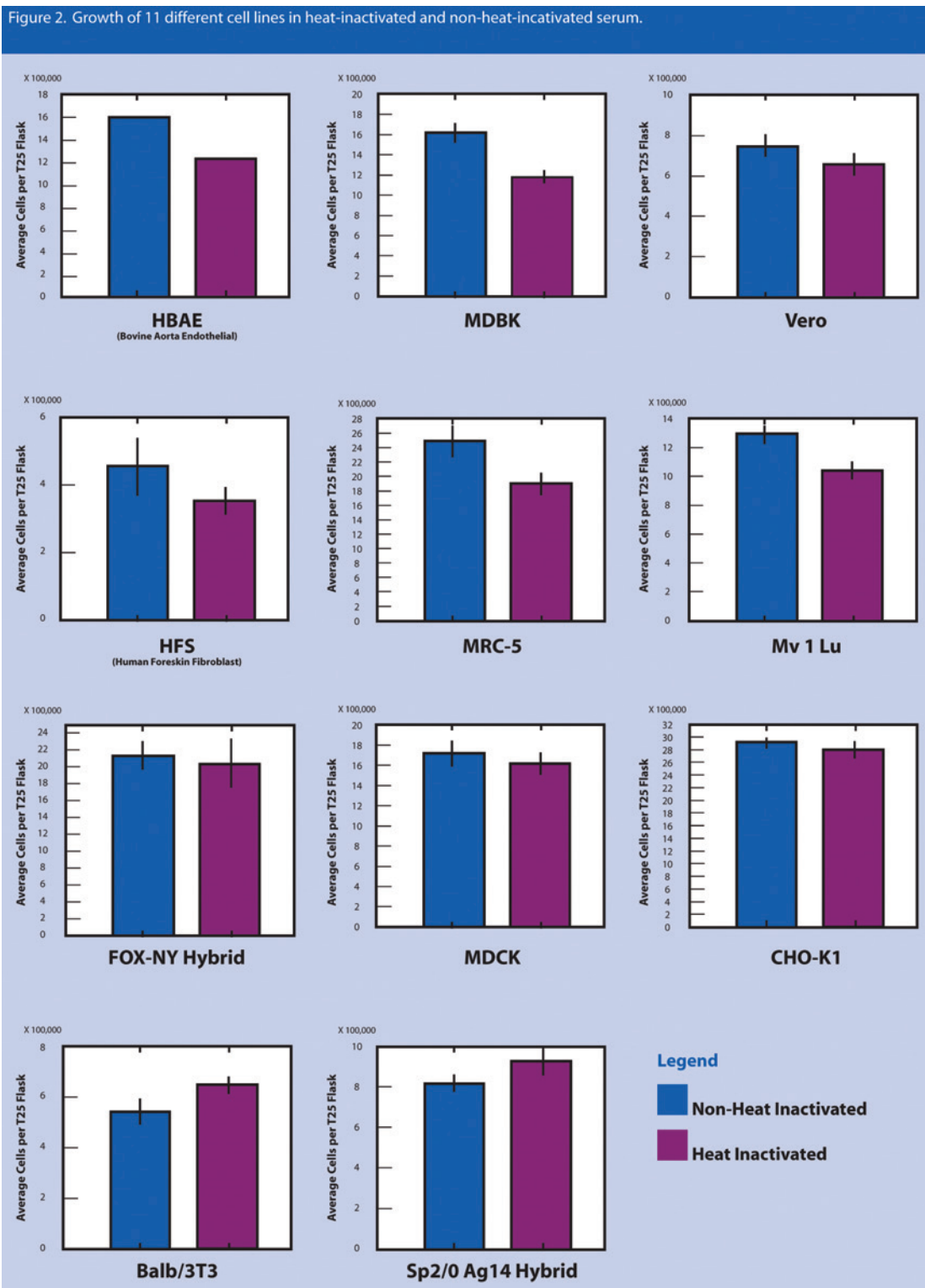
We compared the growth of 11 different cell lines in untreated FBS and heat-inactivated FBS (Figure 2, opposite). The growth of six of the cell lines (HBAE, MDBK, Vero, human foreskin fibroblast, MRC-5, and Mv 1Lu) was affected adversely by heat inactivation. The growth of three cell lines (FOX-NY hybrid, MDCK, and CHO-K1) was not affected by heat inactivation. Only two cell lines (Balb/3T3 and Sp2/OAg14 hybrid) demonstrated slightly improved performance in heat inactivated serum. Thus heat inactivation, when performed properly, offers little or no advantages for cell growth and usually results in decreased growth rates.

However, when performed improperly, heat inactivation will almost always adversely affect serum products. The heating of serum products results in the formation of precipitates which are frequently mistaken for microbial contaminants. Many cell culturists notice this cloudiness and will further incubate the entire contents of the bottle at 37°C in an attempt to "culture" the microorganism. Unfortunately, this exacerbates

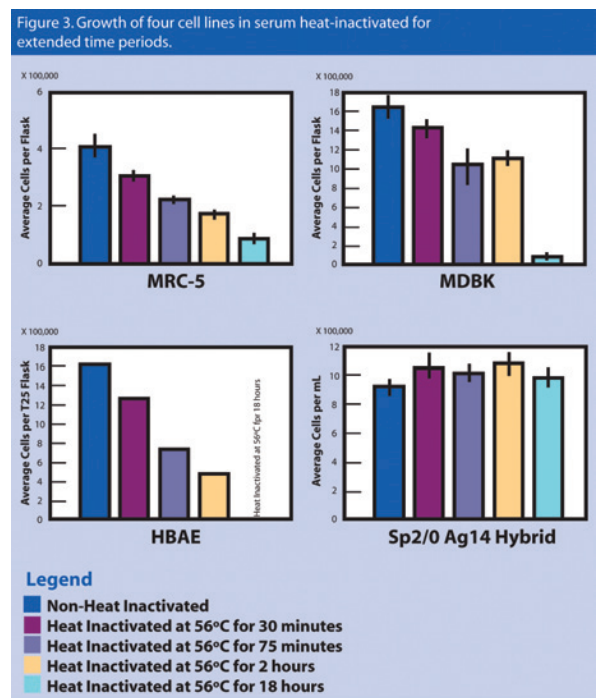
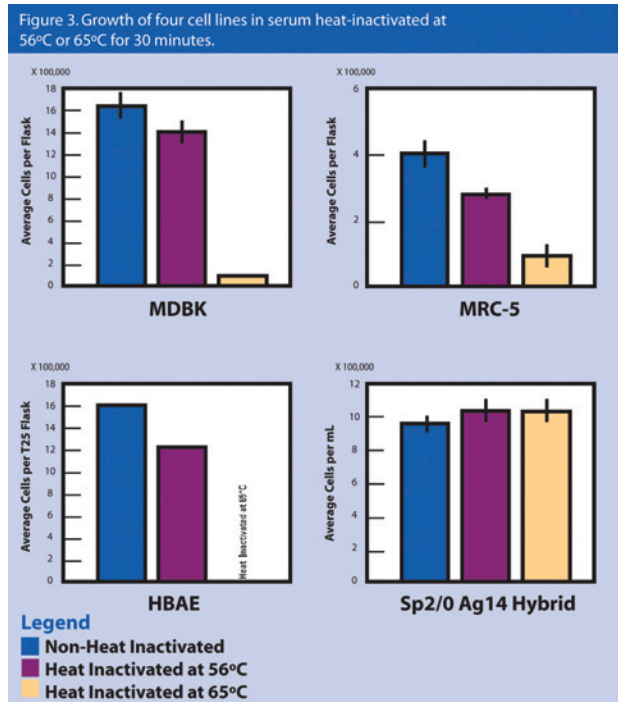
the problem since prolonged incubation only results in the formation of additional precipitate. This precipitate resembles a microbial contaminant to the extent that even experienced cell culturists and microbiologists often cannot distinguish between the two. Extensive testing (electron microscopy, plating on bacterial growth media, Gram staining, etc.) is required to confirm the presence of a precipitate. These tests waste considerable time and resources simply to convince cell culturists that the serum is not contaminated with bacteria. Precipitates are also more likely to form if serum is heat inactivated at temperatures above 56°C or for more than 30 minutes or if the contents

of the bottle are not properly mixed.

Although 56°C is the most common temperature for heat inactivation, some protocols list higher temperatures, which further compromise the growth-promoting ability of serum, in addition to resulting in the formation of additional precipitates. The growth of MRC-5 and MDBK cells decreases dramatically in FBS that has been heat inactivated at 65°C for 30 minutes (Figure 3). FBS heat inactivated at 65°C fails to support the growth of HBAE cells, even though the growth of Sp2/0 Ag14 hybrid cells is not affected.



Serum products are often exposed to high temperatures for more than 30 minutes, either intentionally or unintentionally, e.g., serum is mistakenly left in the water bath overnight. Extended heat inactivation also increases the formation of precipitates and decreases the growth promotion capacity of the serum. Figure 4 shows the growth of HBAE, MRC-5, MDBK, and Sp2/0 Ag14 hybrid cells in serum that has been heated at 56°C for 30, 75, 120, and 1,080 minutes. The growth of HBAE, MRC-5, and MDBK cells is affected adversely with each increase in exposure time. The HBAE cell line is very sensitive to heat inactivated serum and failed to grow in FBS heat inactivated for 1,080 minutes. The growth of MRC-5 and MDBK cells was also impaired significantly, although to a lesser degree than the HBAE cells. Again, as in the exposure to higher temperatures, the growth of Sp2/0 Ag14 hybrids did not seem to be affected by increased exposure times.



Several factors can influence the total time that serum is exposed to high temperatures. Glass and plastic (PETG) differ in their heat capacities, which directly affects heating rates. To reduce breakage and facilitate storage, plastic bottles have largely replaced borosilicate glass bottles. These plastic bottles increase the time required for the contents to reach 56°C by approximately 30 percent. This insulating effect also increases the amount of time required for the contents of plastic bottles to cool following heat inactivation. The cooling rate also depends on whether

Figure 5. Cooling Rates of Serum in ice baths or refrigerator.

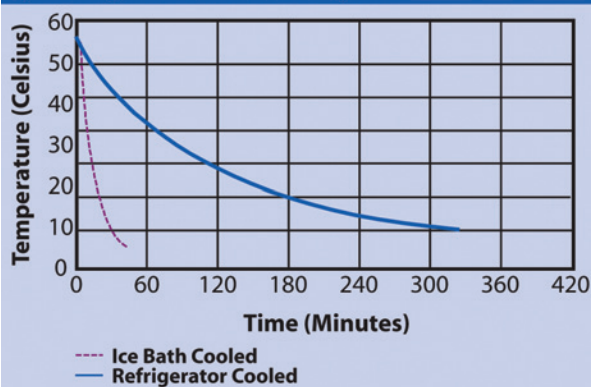
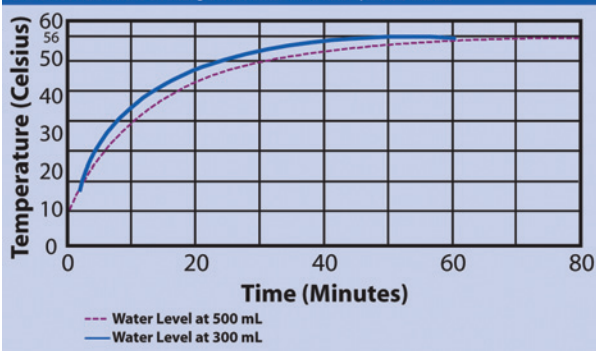


Figure 6. Heating rates in baths in which water levels were equal to either the 300 mL or 500 mL graduation of a standard plastic bottle.



the serum is placed into a refrigerator, freezer, or ice bath following heat inactivation (Figure 5). Heat inactivated serum required 30 minutes to cool to a temperature of 10°C in an ice bath versus 330 minutes when simply placed into a refrigerator. Dividing the serum into aliquots facilitates heating and cooling. The water level in the heat inactivation bath will also affect heating rates of the serum. It is often inconvenient to fill the water bath such that it equals the level of serum in the bottle since the bottles will have a tendency to float. Floating can be prevented by placing commercially available lead weights around the necks of the bottles. Figure 6 illustrates the heating rates in water baths in which levels were equal to either the 300mL or the 500mL graduation of a standard plastic bottle. At the 500mL graduation, the serum required 40 minutes to reach 56°C. At the 300mL graduation, the serum required 60 minutes to reach 56°C thus increasing the total exposure time to elevated temperatures.

In summary, most cell culture applications do not require heat inactivation of serum. In most cases, heat inactivation does not improve the growth promotion capability of the serum and may actually have adverse effects. In the few instances in which heat inactivation improved performance, the improvement was minimal. Moreover, heat inactivation causes an increase in the formation of precipitates which are frequently mistaken for a microbial contaminant. This creates unnecessary concerns and also consumes valuable resources on behalf of the user as well as the supplier.

Customers who currently heat inactivate their serum should determine if this procedure is really necessary with their particular cell lines or culture systems. Serum is often inadvertently heated for longer periods of time and at higher temperatures than recommended in traditional protocols. If heat inactivation is necessary, it should be strictly monitored and performed using a procedure that is repeatable.

References:

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