A Practical Guide to ISO 10993-5: Cytotoxicity

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ISO 10993

Required for all types of medical devices, cytotoxicity testing is a key element of the international standards.

The international standards compiled as ISO 10993, and the FDA blue book memorandum (#G95-1) that is based on 10993-1, address the critical issue of ensuring device biocompatibility by identifying several types of tests for use in selecting device materials. Required for all types of devices, cellular toxicity testing is covered in 10993-5: "Tests for Cytotoxicity—In Vitro Methods." This standard presents a number of test methods designed to evaluate the acute adverse biological effects of extractables from medical device materials. In performing these tests, laboratory technicians culture mammalian cells, usually of mouse or human origin obtained from a commercial supplier, in flasks using nutrient culture media. (The Latin term \textit{in vitro} refers to the culturing of cells "outside of the body," or literally "in glass.") The lab techniques involved are much like those used to grow bacteria. Cultured mammalian cells reproduce by cellular division and can be subcultured to produce multiple large flasks of cells for use in evaluating materials. This article in MD&DI’s continuing series on ISO 10993 provides an overview of cytotoxicity testing and discusses the benefits of performing such procedures.

TEST METHODS

In standard cytotoxicity test methods, cell monolayers are grown to near confluence in flasks and are then exposed to test or control articles directly or indirectly by means of fluid extracts. In the elution test method, which is widely used, extracts are obtained by placing the test and control materials in separate cell culture media under standard conditions (for example, 3 cm$^2$ or 0.2 g/ml of culture medium for 24
hours at 37°C). Each fluid extract obtained is then applied to a cultured-cell monolayer, replacing the medium that had nourished the cells to that point. In this way, test cells are supplied with a fresh nutrient medium containing extractables derived from the test article or control. The cultures are then returned to the 37°C incubator and periodically removed for microscopic examination at designated times for as long as three days. Cells are observed for visible signs of toxicity (such as a change in the size or appearance of cellular components or a disruption in their configuration) in response to the test and control materials (Figures 1 and 2).

Figure 1. A confluent monolayer (100 x magnification) of well-defined L929 mouse fibroblast cells exhibiting cell-to-cell contact. This appearance is indicative of a noncytotoxic (negative) response in the elution test method.

Figure 2. L929 mouse fibroblast cells (100 x magnification) that illustrate a positive cytotoxic reaction in the elution test method. The cells are grainy and lack normal cytoplasmic space; the considerable open areas between cells indicate that extensive cell lysis (disintegration) has occurred.

Alternatively, samples of test and control articles can be applied directly to monolayers of cells covered with nutrient medium or to a semisolid, nutrient agar overlayer that cushions the cells from any physical effects that may be caused by contact with the samples. During the subsequent incubation period, extractables from the samples will migrate into the nutrient medium or through the nutrient agar overlay to the underlying cells. After incubation, the monolayers are evaluated in terms of the presence or absence of a zone of cellular effects beneath and surrounding the sample (Figure 3). Extraction conditions in the overlay and direct contact methods are less rigorous than in the elution test. However, these methods are particularly useful if only very small quantities of samples are available or when only one surface of a material needs to be evaluated.

Figure 3. An agar diffusion flask containing a sample of positive control material. The discoloration that extends outward from the material indicates that the presence of the sample has caused the
cells to lyse, losing the vital stain incorporated in the agar layer.

The elution and direct contact and overlay methods are described in the technical literature and in the U.S. Pharmacopeia as well as in ISO 10993-5. Two additional methods, although used much less frequently than the techniques mentioned above, are used sufficiently to require mention. In the inhibition-of-cell-growth method, saline extracts are added to cell suspensions, and the inhibitory effects on the cells of the extracts are determined by measuring the cell mass after a standard period of incubation. In the Japanese colony-forming assay, a specified number of cells are exposed to an extract from the test article or control; then, following incubation, colonies of cells are counted for the test and control samples. If fewer colonies are formed with the test-article extract than with the negative control, this is taken as evidence of cytotoxicity.

**BENEFITS OF CYTOTOXICITY TESTING**

Cytotoxicity testing is a rapid, standardized, sensitive, and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractables. The high sensitivity of the tests is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body. A mammalian cell culture medium is the preferred extractant because it is a physiological solution capable of extracting a wide range of chemical structures, not just those soluble in water. Antibiotics can be added to the medium to eliminate potential interference from microbial contamination that may be present on the test material and control samples. Results of cytotoxicity tests correlate reasonably well with short-term implant studies. However, they do not necessarily correlate well with other standard tests of biocompatibility that are designed to examine specific end points (such as sensitization) or that use extracts prepared under more rigorous conditions (for example, at 121°C in saline or cottonseed oil).

Cytotoxicity test methods are useful for screening materials that may be used in medical devices because they serve to separate reactive from nonreactive materials, providing predictive evidence of material biocompatibility. The ISO 10993-1 standard, "Guidance on the Selection of Tests," considers these tests so important that they are prescribed for every type of medical device, along with sensitization and irritation testing. Cytotoxicity test methods are also useful for lot-to-lot comparison of materials, for determining whether a potential replacement material is equivalent to that currently being used, and for troubleshooting and exploring the significance of changes in manufacturing processes.

**CONCLUSION**

Testing for cytotoxicity is a good first step toward ensuring the biocompatibility of a medical device. A negative result indicates that a material is free of harmful extractables or has an insufficient quantity of them to cause acute effects under exaggerated conditions with isolated cells. However, it is certainly not, on its own merit, evidence that a material can be considered biocompatible—it is simply a first step. On the other hand, a positive cytotoxicity test result can be taken as an early warning sign that a material contains one or more extractable substances that could be of clinical importance. In such cases, further
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investigation is required to determine the utility of the material.

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