TECHNOLOGY FEATURE

REPRODUCIBILITY: RESPECT YOUR CELLS!

Numerous variables can torpedo attempts to replicate cell experiments, from the batch of serum to the shape of growth plates. But there are ways to ensure reliability.

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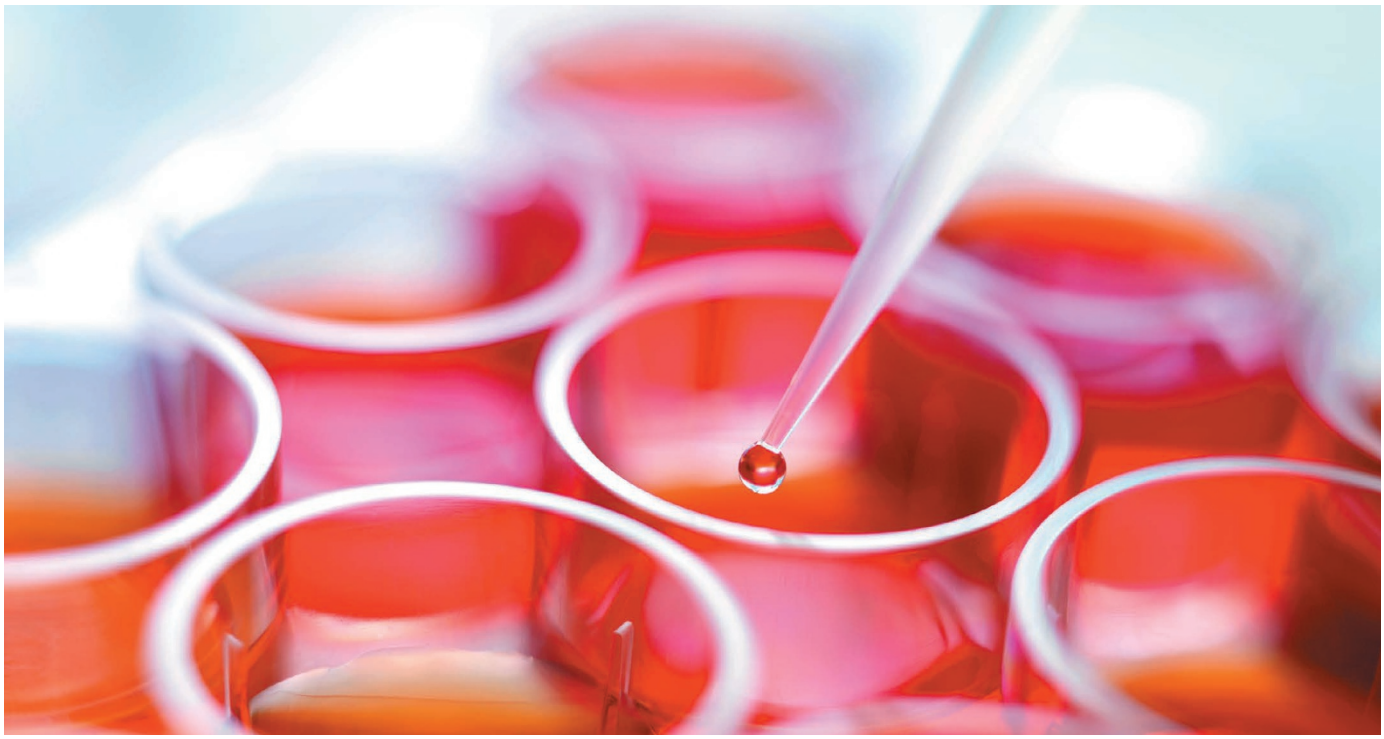


Subtle aspects of cell culture can wreck results. Researchers should check cell identity and behaviour, and carefully characterize reagents.

BY MONYA BAKER

When Alastair Khodabukus tried to engineer muscle fibres in his new laboratory, he saw something strange: the tissue was convulsing. He had been growing fibres from the same mouse-derived clone for years, but these were different. They burned more glucose, contained lower amounts of a protein that promotes faster relaxation and fatigued less readily than those he had grown before his lab moved from Dundee, UK, to the University of California, Davis. The difference, he thinks, was due to how cows are raised in the United States[1].

Most academic labs culture cells by using fetal bovine serum (FBS), a liquid extracted from clotted cow blood and collected from abattoirs when pregnant cows are slaughtered. What ends up in the serum depends on factors such as diet, geographical location, time of year, whether the animals receive hormones or antibiotics and the gestational age of fetal calves. Substantial amounts of FBS are added as a supplement to the culture media in which cells grow; 5–15% of the volume of growth media is typical. FBS composition can affect how thick an engineered tissue becomes, cause spontaneous artefacts that mimic cell activity and even influence how surface receptors respond to a given compound. "FBS is like a big dark cloud over our heads, not knowing what's real and what's not," says Khodabukus, now a postdoctoral researcher at Duke University in Durham, North Carolina.

And serum is just one of many factors that researchers have to consider when studying cells. At a US National Institutes of Health (NIH) workshop on cell culture and reproducibility last year, Richard Neve, a cancer biologist at the biopharmaceutical company Gilead Sciences in Foster City, California, worried that researchers could become overwhelmed. "A lot of labs see the magnitude of the problem and the complexity of the problem, and enter the primordial part of their brain and shut down." With the right mindset, however, and some obsessive checking and planning, researchers can gain confidence in performing their experiments.

The most basic step is to ensure cells' genetic identity. Journals and funders now ask researchers to disclose whether they have checked to make sure that, say, cell lines representing corneal or skin tissue are not actually a fast-growing line derived from human cervical cancer. But cells' behaviour can also change with density, proliferation rates, growth media, the presence of contaminants and the time kept in culture[2].

Serum is arguably the most common supplement in cell-culture media, and also the least consistent. Human serum harbours thousands of distinct proteins originating from a wide range of cells and tissues, as well as thousands of small-molecule metabolites, all in varying concentrations. FBS probably has similar complexity, with plentiful factors to support a fast-growing fetus, too.

FBS is not only variable, it also differs from the fluid that cells are exposed to in their natural environment. Most cells are in contact not with blood directly but with the interstitial fluid that bathes organs, says Adam Elhofy, chief science officer at Essential Pharmaceuticals in Ewing, New Jersey, a company developing a serum replacement for multiple cell types. Hormones, growth factors and other signalling molecules are abundant in serum, but tightly regulated in organs, he says (see 'Bovine serum's wide range').

GOING SERUM-FREE

To overcome such concerns, reagent firms have developed serum-free growth media. Scientists pursuing 'bioprocessing' applications — such as the manufacture of therapeutic proteins and vaccines, a process in which animal products are frowned on — have embraced the serum-free alternative. Stem-cell researchers, who know these cells are sensitive to even small changes in growth conditions, are also enthusiasts.

Many more researchers are now beginning to pay attention to how they treat their cells, driven by concerns about consistency and a push into translational medicine. These priorities are encouraging more scientists to avoid serum, says Ken Yoon, who is head of strategic marketing in the research division of MilliporeSigma, a life-science reagents company in Billerica, Massachusetts. Chemically defined, serum-free media is one of the fastest growing segments in the cell-culture space, he adds.

But serum-free media are not always possible, or pragmatic. "Everyone agrees it would be a great thing if we can move away from FBS and to something more defined," says Jon Lorsch, head of the US National Institute of General Medical Sciences in Bethesda, Maryland. "The question is how feasible it is, and we don't know the answer to that question."

Most serum-free formulations apply only to a specific cell type or closely related group of cell lines. Vendors sell one serum-free medium for, say, Chinese hamster ovary cells, an epithelial cell line that is often used to produce therapeutic proteins, and others to expand particular types of blood cells. Formulations don't work for all cell types: many 'primary cells' — those taken directly from living tissue — require serum to grow after they are removed from the cues that the body provides, says Jennifer Welser-Alves, associate director of research and development at ScienCell Research Laboratories in Carlsbad, California. "Anything you can do to boost the cells and keep them growing is necessary," she says. Some formulations require adding just 2% FBS to primary growth media, a low volume of serum that helps cut down on variability.

Even if the option is available, many researchers are unwilling to take the time, or the risk, to wean their cells off serum, says Paul Price, a culture-media consultant in Mount Pleasant, South Carolina, who has designed serum-free formulations. "Every year since 1980, people have been saying that serum is dead," he says. "Serum is still very popular because people like the idea that they can grow cells and not have fabulous technique." Culture is tough on cells: researchers pipette them from dish to dish, freeze and thaw them, add digestive enzymes to detach them from substrates and more. Serum is a balm for such abuses, says Price.

STUCK WITH SERUM

No commercial formulation is available for skeletal muscle fibres, says Khodabukus. He has spent two years tinkering with recipes that combine dozens of growth factors and other signalling molecules. When the fibres' performance changes with each lot of serum, it disrupts his own projects and muddies collaborations, he says. "I'm going to spend the rest of my life working with this system, and as a scientist I want control. If we can get this to work and be consistent, we can get this to work in every lab around the world."

Keith Baar, Khodabukus's former postdoc adviser at the University of California, Davis, relies on a more common solution: he keeps a freezer in his lab that's dedicated to storing serum. When serum starts to run low, he orders and tests at least four batches, and watches the cells' performance to find the closest match to that in his current experiments. Then he buys 100 bottles from the same lot of serum. That can drain US$25,000 from his lab budget, but it means that his lab members can continue their experiments without stopping every few months to test more lots of serum.

> *"One of the hardest things to assess is what constitutes a healthy cell."*

Researchers who don't test their serum could run into trouble, says Matthew Sikora, a cancer biologist at the University of Colorado, Denver. He uses breast cancer cell lines to work out the effects of 'weak oestrogens', which include certain drugs and industrial chemicals such as bisphenol A. Sikora buys serum that has been treated with charcoal to strip out steroid hormones and other greasy molecules. Then he tests the serum on cells that have or lack oestrogen receptors; if the hormones in the serum have been effectively removed, the proliferation rates should be the same.

Last year, he and others in his laboratory were stalled for about six months when sequential batches of serum failed this initial screen. Differing hormone content "totally flipped" the interpretation of how a cancer drug worked[3]. Sikora thinks that unrecognized variation in serum might explain why he and a potential collaborator could not get consistent results.

But even when researchers do batch testing, they don't always know what to look out for. Some laboratories simply buy the serum lot in which their cells grow the fastest. Instead, they should tailor screens to the intended study. Researchers also need to report exactly how they screen serum to enable others to reproduce the work, says Sikora.

Some cells and experiments will be more sensitive to the effects of serum than others. The 'transformed' cell lines selected over decades for robust growth tend to vary less than 'diploid' lines or primary cell lines that more closely resemble natural tissue.

Researchers always need to be careful, says Mariella Simon, a cell and developmental biologist at the Children's Hospital of Orange County in California. Ideally, they should have enough serum to last an entire study. And when they do move to a new bottle, they should make sure that no other reagents have changed and that they have enough old serum stockpiled to test whether any strange results can be attributed to the switch. It is easy, for example, to conclude that something is going wrong with a protocol to introduce DNA into cells when, in fact, a new batch of serum has affected division rates. Researchers should also record the information supplied by vendors about serum, including lot numbers, says Simon. "You can't just use your labmate's serum that might have been aliquoted a long time ago and labelled FBS."

BOVINE SERUM'S WIDE RANGE

The bioactive compounds in fetal bovine serum can vary dramatically from lot to lot. Selected components are shown below.

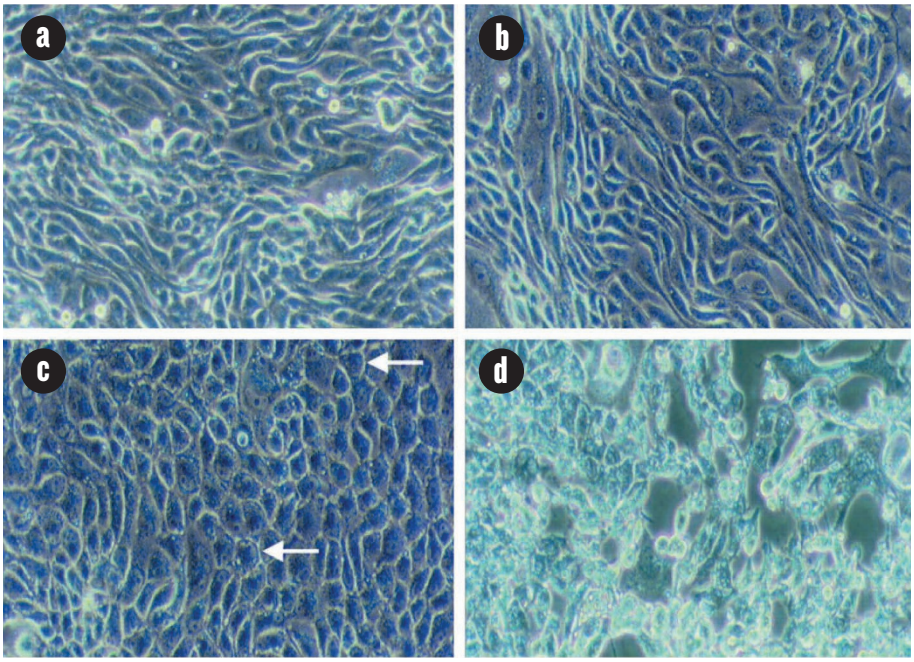
Components	Average (range)
Endotoxin	0.356 ng ml^{-1} (0.008–10.0)
Total protein	3.8 g dl^{-1} (3.2–7.0)
Alkaline phosphatase	255 mU ml^{-1} (111–352)
Lactic dehydrogenase	864 mU ml^{-1} (260–1,215)
Cortisol	0.5 µg dl^{-1} (<0.1–2.3)
Insulin	10 µU ml^{-1} (6–14)
Parathyroid hormone	1,718 pg ml^{-1} (85–6,180)
Progesterone	8 ng dl^{-1} (<0.3–36)
Testosterone	40 ng dl^{-1} (21–99)
Prostaglandin E	5.91 ng ml^{-1} (0.5–30.5)
TSH	1.22 ng ml^{-1} (<0.2–4.5)
FSH	9.5 ng ml^{-1} (<2–33.8)
Growth hormone	39.0 ng ml^{-1} (18.7–51.6)
Prolactin	17.6 ng ml^{-1} (2.00–49.55)

TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone

SOURCE: P. J. PRICE & E. A. GREGORY *IN VITRO* **18**, 576–584 (1982)

Contaminants can confound experiments, too. One of the most insidious is *Mycoplasma*. This tiny bacterium can slip through sterilizing filters and is unfazed by many antibiotics. It depletes cells' nutrients and alters DNA and protein synthesis. An analysis of nearly 10,000 rodent and primate samples found that more than 10% contained RNA sequences unique to *Mycoplasma*[4]. Conventional *Mycoplasma* testing can take several weeks and still miss rare strains, but PCR-based tests are now providing swifter, surer answers, says Yvonne Reid, who leads standards-setting efforts at American Type Culture Collection, a non-profit repository for cell lines in Manassas, Virginia.

To avoid serum contamination, some researchers are opting for gamma irradiation. Several common contaminants, including *Mycoplasma*, are sensitive to even low levels of radiation. But this requires a balancing act: radiation also damages growth proteins and bioactive molecules that help cells thrive. Many vendors offer gamma-irradiated serum, and the International Serum Industry Association has set up a working group to elucidate its effects[5]. Cell-culture consultant Raymond Nims of RMC Pharmaceutical Solutions in Longmont, Colorado, advises anyone who plans to work with gamma-irradiated serum to first test that cells perform as expected, and to remember that even contaminant-free serum cannot prevent infection by other sources.



Epithelial cells growing in regular culture medium (a, at 6 hours; b, at 48 hours) become rounded in the presence of cholesterol (c, shown by arrows) and shrivel and die when cholestane is added (d)[7].

ERRORS COME FROM EVERYWHERE

The cells' physical environment is a profound influence. Researchers at the Wyss Institute in Boston, Massachusetts, found that mechanical peristalsis-like deformations and fluid flow changes alone could, without any alterations to the growth media, induce functional villi from cells that otherwise grow flat[6].

Lab dishes of different brands leach different chemicals into cell-culture media, and can confound studies of cell metabolites. Deliberate additives can change cell metabolism in unappreciated ways: antibiotics in particular frequently impair mitochondrial activity. Even a glass door on a lab refrigerator can ruin experiments, because some chemicals in growth media are sensitive to light. Just changing the laboratory plates, and thus the height of media in which cells are sitting, can alter how cells behave. What's more, cells growing in a given culture are not identical, and the subset of cells that thrives the most can quickly dominate a population. That means cells may not revert back to former behaviour if a researcher decides to restore previous experimental conditions.

In all these experiments, the cells themselves are the most important variable. There is no quick, simple way to know that cells are fit for purpose, says John Masters, a cancer researcher at University College London, and author of cell-culture reference books. "Get to know your cells," he urges. "The best assay you have for knowing how happy the cells are is looking at them."

Leland Foster, a cell-culture consultant in Salt Lake City, Utah, and former chief executive of HyClone Laboratories, the cell-culture reagents company now owned by GE Healthcare Life Sciences, thinks that trainees cannot, generally, be expected to take the care required. "One way that labs could get away from variability is having some expertise that is resident in that laboratory," he says. Growing cells is, he says, best left to "an expert cell culturist" who can tell when cells are "smiling or frowning", and who will ask serum and other vendors tough questions about the products they buy for their cells.

Cells react differently when they are growing rapidly or persisting in a stationary phase. If they are 'overpassaged' (that is, kept in culture too long), other changes can occur and affect reproducibility. Even when the genetic identity of a cell line has been authenticated — as is now broadly recommended — other crucial attributes, such as the growth state, number of doublings and checks for contamination, too often remain undocumented. "Authentication means more than identity," Reid says. Researchers hoping to reproduce experiments should not have to "act like a detective" to work out what state the cells were in when building on a reported study.

Given these unknowns, researchers should take a week or so to optimize their cells' growth and plot a growth profile before launching experiments, says Reid, who is coordinating an open-access series about best practices in cell culture. A growth profile can inform researchers when to harvest cells, when to do assays and when to go back to a distribution bank for a fresh batch of cells. It can also warn scientists if they are overlooking important variables. Most of all, researchers must be alert and creative to make sure the cells they are using are consistent across a study, Neve says. "There is no single set of experiments that works for everyone."

Concrete data, like good microscope images or expression data, can help researchers recognize when the cells used in their experiment have changed, says Anne Plant, a division chief at the US National Institute of Standards and Technology in Gaithersburg, Maryland, who hopes to find quantitative ways of making cell-culture experiments comparable across laboratories. "One of the hardest things to assess is what constitutes a healthy cell," she says.

Even harder can be the consequences for researchers who neglect to think of cells as "live beings that need to be looked after and cared for", says Masters. "Someone's PhD goes down the pan, or a grant is lost, or years of work are wasted because they are not doing fairly simple quality control." ■

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