Connecting with Neuroscience

Combinations of GIBCO™ Serum-Free Media Improve Growth of Primary Neurons, Facilitating Better Control in Research Studies

In 1907, conducting a pioneering experiment that marked the dawn of cell culture, Ross Harrison grew frog embryo neural tissue *in vitro*, and demonstrated that each nerve fiber is the outgrowth of a single nerve cell.

Nearly a century later researchers are able to study individual neurons and synapses far more easily thanks to the relatively recent ability to grow primary neurons in culture at low seeding densities.

Experience has shown that neurons grown in GIBCO™ media optimized for their survival facilitate better control in studies of neuronal development, mechanisms of neuronal signaling, electrophysiology, pharmacology, plasticity, *in vitro* growth requirements, gene expression, and neurotoxicity (1).

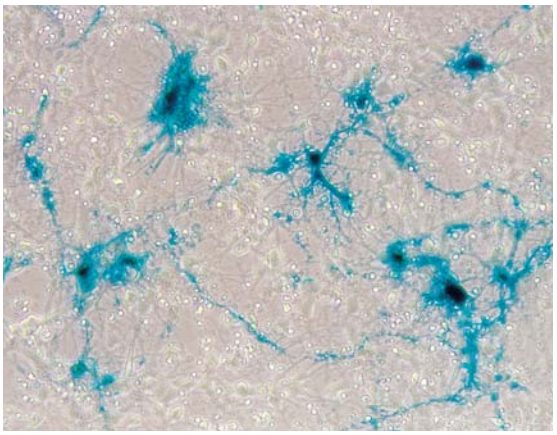
Invitrogen, under the GIBCO™ brand name, offers an ever-expanding array of cell culture media, supplements, and growth factors specifically developed to advance neuroscience research.

Neurobasal™ Media

Neurobasal™ Media are basal media formulated to meet the neural cell's special requirements. When supplemented, they allow for the long-term maintenance of the normal phenotype and growth of neural cells, and maintain nearly pure populations of neural cells for up to 10 days without the need for an astrocyte feeder layer.

Neurobasal™ Formulations

Neurobasal™ Medium is an optimized basal medium developed for low-density plating and long-term viability of fetal hippocampal and other neurons of the central nervous system. It is supplied glutamine-free. Supplemented with serum-free B-27, 25 µM glutamate and 0.5 mM L-glutamine, it provides excellent long-term viability of rat embryonic hippocampal neurons (2), even after four weeks in culture with greater than 90% viability for cells plated at 640/mm^2 and greater than 50% viability for cells plated at 160/mm^2. Glial cell growth at five days is reduced to less than 0.5%, resulting in a nearly pure neuronal population.



Primary rat cortical neurons, E18, were plated in GIBCO™ Neurobasal™ Medium at 2 x 10^5 cells/well in 24-well plates. *Cells were transfected 4 days later using 0.8 µg of pCMV-SPORT β-gal DNA, purified using Concert™ High Purity Plasmid Purification System, and 4 µl of Lipofectamine™ 2000 Reagent.*

In addition to supporting low or high density growth of fetal hippocampal neurons, the combination of Neurobasal™ and B-27 has also been shown to support the growth of neurons from embryonic rat striatum, substantia nigra, septum, cortex, and neonatal dentate gyrus and cerebellum (3).

When supplemented with N-2, B-27 or serum, Neurobasal™ Medium is effective in the growth of tumor cell lines of neuronal origin. Because it does not contain the excitatory amino acids glutamate and aspartate, the medium is amenable to the study of these neurotransmitters.

Neurobasal™-A Medium

The growth of adult CNS neurons requires gentle separation of their numerous connections, a density gradient for the separation of oligodendrocytes and enrichment of neurons, an adequate substrate for attachment, and a dedicated medium for growth.

Properly supplemented, Neurobasal™-A Medium is the medium of choice for hippocampal and other postnatal and adult neurons of the central nervous system.

With adequate isolation methods, Neurobasal™-A Medium supplemented with B-27 permits the isolation of spherical remnants of hippocampal neurons from any age rat, and promotes the regeneration of axon and dendrite-like processes. A neuron-like morphology is maintained for several weeks in culture. The addition of β-FGF enhances viability at least three-fold, independent of age, without affecting the process.

Neurobasal™ Medium without Phenol Red and Neurobasal™-A Medium without Phenol Red

These basal formulations are available for research on specific receptors such

continued on page 3

continued from page 2

as estrogen, for downstream protein purification, or for other studies in which the presence of phenol red is undesirable.

B-27 Supplements

B-27 is an optimized serum substitute developed for low-density plating and long-term viability and growth of hippocampal and other CNS neurons. When added to Neurobasal™ Media, B-27 supports the growth of neural cells without an astrocyte feeder layer, and is effective for the growth of neural tumor cell lines.

B-27 Supplement Minus AO is the complete B-27 Supplement without five antioxidants (vitamin E, vitamin E acetate, superoxide dismutase, catalase, and glutathione) that would interfere with studying free-radical damage to neurons.

B-27 Supplement Minus Retinoic Acid is available for those who are culturing neural stem cells or neurospheres, and need to control the amount of vitamin A present in cultures.

N-2 Supplement

A chemically-defined additive for Neurobasal™ Media, N-2 is recommended for growth of rat primary embryonic neurons and embryonic CNS progenitor cells.

G-5 Supplement

A chemically-defined additive for the growth of glial cells, G-5 also supports growth of primary and serial tumor lines of the astrocytic phenotype when added to D-MEM, D-MEM/F12, E-MEM, or other basal formulations.

References

1. Price, P.J., Brewer, G.J. Serum-free Media for Neural Cell Cultures, Adult and Embryonic. *Protocols for Neural Cell Culture*, 3rd Ed., Humana Press, Inc. (2001)

2. Brewer, G.J., Toricelli, J.R., Evege, E.K., Price, P.J. Optimized Survival of Hippocampal Neurons in B-27 Supplemented Neurobasal™, a New Serum-free Media Combination. *J. Neurosci. Res.* 35:567-576 (1993)

3. Brewer, G.J. Serum-free B-27/Neurobasal™ Medium Supports Differentiated Growth of Neurons from the Striatum, Substantie Nigra, Septum, Cerebral Cortex, Cerebelum and Dentate Gyrus. *J. Neurosci. Res.* 42:674-683 (1995)

Related Reading

Ohki, E.C., Tilkins, M.L., Ciccarone, V.C., Price, P.J., Improving the Transfection Efficiency of Post-mitotic Neurons. *Journal of Neuroscience Methods*. 112:95-99 (2001)

Neuronal Cells

MEDIUM	CELLS	APPLICATIONS
Neurobasal™ Medium (1X), liquid Neurobasal™ Medium (1X), liquid without phenol red Neurobasal™-A Medium (1X), liquid Neurobasal™-A Medium (1X), liquid without phenol red	Fetal neurons Adult and post natal neurons (>1 week old)	Basal medium lacking excitatory amino acids used in conjunction with serum-free medium. Long-term growth of neurons
with B-27 Supplement (50X), liquid or with B-27 AO Supplement (50X), liquid or with B-27 AO Supplement Minus Retinoic Acid (50X), liquid	Primary rat embryonic hippocampal neurons; primary rat neurons from striatum, substantia nigra, septum; neural stem cells	Growth and maintenance. Minimizes glial cell proliferation. B-27 AO to study free-radical damage, apoptosis. (B-27 AO is B-27 without any antioxidants.) B-27 Minus Retinoic Acid for neural stem cell studies
with N2 Supplement (100X), liquid	Primary rat embryonic hippocampal neurons, tumor cell lines of neuronal origin (PC12, B104, N1E-115 and NS20); neural stem cells	Maintenance of primary neurons (low protein, <125 µg/ml). Growth and maintenance of neuronal tumor cell lines and for neural stem cell studies
with G5 Supplement (100X), liquid	Primary glial cells, tumor cell lines of glial origin (U-251, MGsp, C62BD, RN-22), astrocytes, microglia, oligodendrocytes	Growth and maintenance of cell lines of astrocytic origin

For more information on neurobiology products, see Chapter 3 of the GIBCO™ 2002 catalog.

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TOOLS

NEUROBASAL™ MEDIUM / B27 SUPPLEMENT: A NEW SERUM–FREE MEDIUM COMBINATION FOR SURVIVAL OF NEURONS

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Growth of neurons occurs by process outgrowth. Many neurons die during this differentiation. The surviving neurons maintain unfragmented processes. B27 was developed as a supplement to D–MEM for the growth of hippocampal neurons by optimization of over 20 components of a previously published serum-free supplement called B18 (1). With rat embryonic hippocampal neurons, D–MEM with B27 produced a 4–day neuron survival above 50%, independent of plating density above 160 plated cells / mm^2 (2). However, the combination of B27 with D–MEM or D–MEM:F12 for neuron survival has several problems including, rapid deterioration of the ability of D–MEM:F12 to support neuron survival and the presence of potential excitotoxic amino acids in both basal media. Basal media (including D–MEM and D–MEM:F12) were developed for rapid cell division of somatic cells, so it is not surprising that these media are not optimal for the survival and differentiation of neurons.

Since freshly made D–MEM:F12 produced higher neuron survival than D–MEM in our studies, Neurobasal medium was developed by optimizing concentrations of the components found in D–MEM:F12 but absent from D–MEM: alanine, asparagine, cysteine, glutamate, proline, and vitamin B12 (2). In addition, osmolality, glutamine, and sodium bicarbonate concentrations were optimized. Neurobasal medium, therefore, is a modified D–MEM:F12 with a lower concentration of several amino acids. In addition, ferrous sulfate and the excitatory amino acids glutamate and aspartate were eliminated. In Neurobasal medium with B27, excellent long–term survival was achieved after 4 weeks in culture with greater than 90% viability for hippocampal neurons plated at 640 cells / mm^2 and greater than 50% viability for cells plated at 160 / mm^2 (2). This paper shows the improved performance of Neurobasal medium with B27 over D–MEM with either B18 supplement, serum, or N2 supplement (3). Also, the stability of B27 supplement, the need for glutamate in the medium, and maintenance of immunoreactivity for two neuronal markers neurofilament and MAP2 in Neurobasal / B27 are examined.

METHODS

Isolation and plating of rat hippocampal neurons. All salt solutions, media, and reagents were from Invitrogen Corporation*. Embryos were recovered by c–section under nembutal anesthetic. Individual cells were isolated by trituration 10 times in 1 ml of Hanks' Balanced Salt Solution (HBSS) without Ca^{++} and Mg^{++} and supplemented with 1.0 mM sodium pyruvate and 10 mM HEPES (pH 7.4) using a 9–inch siliconized pasteur pipet with the tip barely fire polished. Divalent cations were restored by dilution with 2 volumes HBSS with Ca^{++} and Mg^{++} supplemented as above. After allowing nondispersed tissues to settle for 3 min, the supernate was transferred to a 15–ml tube and centrifuged for 1 min at 200 × *g*. The pellet was gently resuspended in 1 ml HBSS per brain and an aliquot added to trypan blue stain for a haemocytometer count. The culture vessels were coated with a 0.05–mg / ml solution (0.15 ml / cm^2 surface area) of cold poly–D–lysine (MW 30,000 – 70,000) and incubated for 1 h or overnight. The poly–D–lysine solution was stored at –20°C in polystyrene tubes and was prescreened for toxicity.

Vessels were washed with sterile, deionized cell culture grade water. Vessels can be stored for up to 2 weeks at 4°C to 10°C in sterile deionized, distilled water. If vessels are stored, remove water ~1 h prior to use. To Neurobasal medium (Cat. No. 21103), add 0.5 mM L–glutamine, 25 µM glutamate, and B27 supplement [2 ml of B27 50X concentrate (Cat. No. 17504) to 100 ml Neurobasal medium]. Cells were seeded at the desired densities. Cultures maintained longer than 4 days should have half the medium changed to Neurobasal / B27 without glutamate on day 4 and then once per week. If the initial culture density is higher than 640 cell / mm^2, the medium should be changed twice a week.

* Invitrogen Corporation acquired Life Technologies, Inc. in 2000.

Immunofluorescence staining. Cells were plated at 400 cells / mm^2 and grown for 5 days in Neurobasal / B27. Neurons were fixed for 30 min in 3.7% formaldehyde in PBS; and rinsed and blocked in 1% BSA, 1% normal goat serum, 0.05% TRITON® X–100. Primary antibodies were added together and incubated overnight with the neurons at 4°C. Rabbit anti–neurofilament 200 (1:50) was detected with rhodamine–conjugated goat anti–rabbit IgG (1:500). Mouse anti–MAP2 (1:200) was detected with fluorescein–conjugated goat anti–mouse IgG (1:100).

RESULTS AND DISCUSSION

A discriminating measure of the ability of culture medium to maintain cell viability is to measure survival at low cell plating densities for periods longer than 3 days. For neurons, this is particularly true due to supposed needs for trophic factors. After 1 day in culture, survival correlated well with the number of cells with processes (data not shown). After 4 days in culture, survival was much more discriminating for the effect of medium components. The 5 day survival of neurons in Neurobasal / B27 was far better than cells cultured in D–MEM with B18 and Neurobasal / N2 at all plating densities (figure 1). At all but the highest plating density, Neurobasal / B27 was superior to Neurobasal medium with 5% FBS.

To examine the stability of B27, neurons were plated at 160 cells / mm^2 in NEUROBASAL medium with freshly thawed B27 or B27 stored at 4°C for 2 months. Four–day survival values were 60% and 64%, respectively. There appears to be no significant loss of activity at 4°C over a period of 2 months. Also, B27 stored at –20°C for 1 year has not shown any loss in neuron survival.

One of the advantages of Neurobasal medium is the omission of the excitatory transmitter amino acids, glutamate and aspartate. Glutamate excitotoxicity is not only a subject of great experimental interest with relevance to hypoxia–ischemia, hypoglycemia, and epilepsy (4), but could be relevant to the ability to maintain neurons in culture. During embryogenesis, the brain capillary endothelium or blood–brain barrier is not as tightly sealed as it is postnatally. Therefore, differentiating neurons with needs for neurite growth may require glutamate at a concentration closer to that found in serum. Adult rat serum glutamate levels are around 11 µg / ml. With serum–free medium, it was possible to optimize the glutamate concentration for survival of embryonic hippocampal neurons. Survival after 4 days indicated an optimum near 25 µM (3.7 µg / ml). Figure 2 examines the effect of the continued presence of glutamate. Neurons originally plated in 25 µM glutamate were either left to grow, or one–third of the medium was changed to fresh medium (D–MEM / B27) with or with-

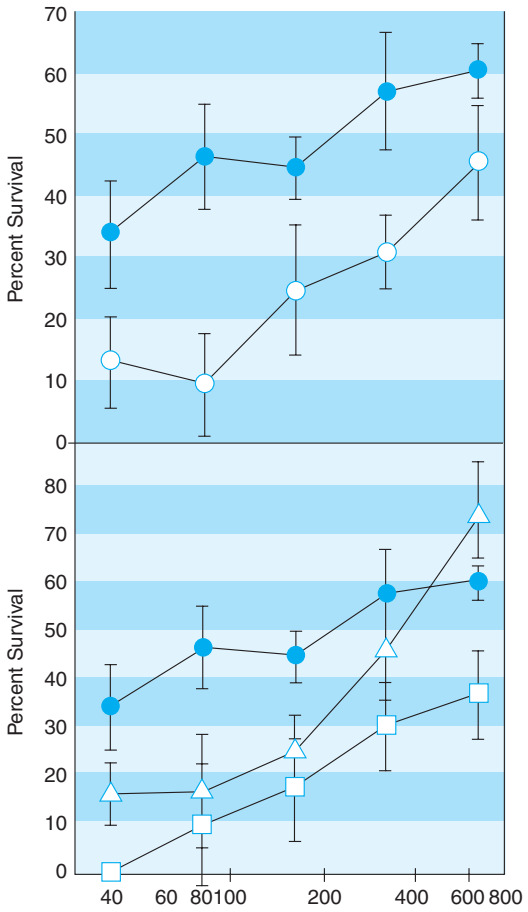

FIGURE 1. Comparison of Neurobasal / B27 to D–MEM and Neurobasal with other supplements. Cells isolated in HBSS from the hippocampus of 18–day–gestation rat embryos were plated at the indicated densities in Neurobasal / B27 (●), D–MEM / B18 (○), Neurobasal / N2 (□), and Neurobasal / 5% FBS (△). After 5 days of growth, live and dead neurons were counted using fluorescein diacetate and propidium iodide as described (2). Survival is the ratio of live to total neurons.

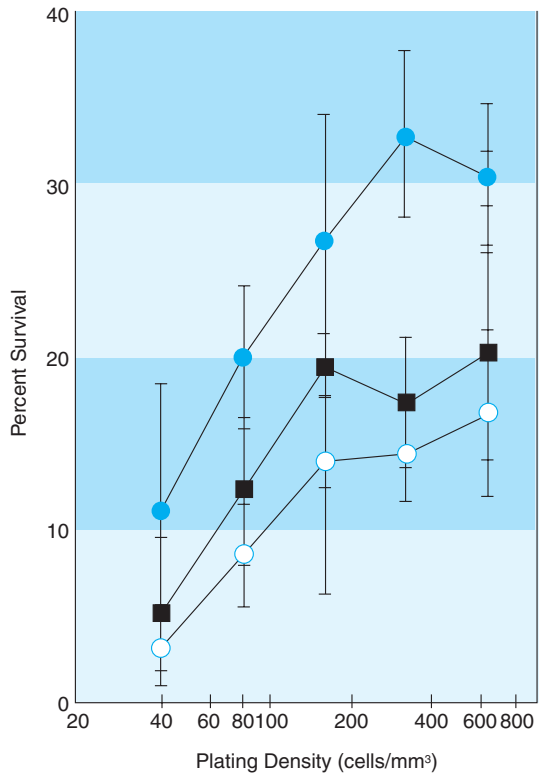

FIGURE 2. The long–term effect of glutamate on neuron survival. Embryonic hippocampal neurons were plated in D–MEM with B27 at the indicated densities. After 4 days of growth, medium was either not changed ( ), or one–third was replaced with fresh medium with ( ) or without ( ) 25 µM glutamate. Survival was determined on day 8.

out 25 µM glutamate after 4 days. Only one–third of the medium was changed to minimize changes in trophic factors present in the conditioned medium. At 320 cells / mm^2, survival that was 60% on day 4 drops to 14% on day 8 without changing the medium. Changing one–third of the medium on day 4 to medium without glutamate increased survival to 33%. The increase in survival was due to fresh medium without glutamate since the survival was not improved by a medium change with glutamate (17% survival). Subsequent tests with Neurobasal / B27 have produced better long–term survivals by replacing one–half of the medium with glutamate–free medium on day 4 (data not shown). Further medium changes depend on cell density: above 160 cells / mm^2, change medium every 3 to 4 days and below 160 cells / mm^2, change the medium once a week.

One– to three–day cultures are characterized by several dendritic processes and a single axonal process (5). Culturing cells for longer periods leads to a more dense network of dendritic processes and continued growth of axons. The cover photograph shows the characteristic features of neuronal cells grown in Neurobasal / B27 for 5 days. Dendritic processes have tapering arbors and frequent branches at acute angles. Axons are identified by their small, uniform caliber and branching at right angles. Dendrites, but not axons, were stained with the microtubule–associated protein MAP2 (yellow / green) (6). Anti–neurofilament was used to stain axons (red), although this cytoskeletal component is not exclusively an axonal marker.

Neurobasal medium with B27 also supported the growth of neurons from embryonic rat striatum, substantia nigra, septum and cortex for 1 week (table 1), as well as dentate gyrus and cerebellum from neonatal rats (Brewer, manuscript in preparation). Even though levels of survival were adequate, further studies are needed to optimize the B27 supplement for each cell type. Recent studies have also shown that adult rat hippocampal neurons can be isolated grown in Neurobasal / B27 (Brewer, manuscript in preparation). This serum–free medium combination was shown to be effective for the growth of tumor cell lines of neuronal origin (B104, PC12). Therefore, support for other CNS primary neurons and neuroblastomas is likely.

In serum–supplemented media, glial cells continue to proliferate which usually necessitates the addition of cytotoxic inhibitors (7) to

TABLE 1. Survival of other rat CNS neurons in Neurobasal/B27.

Cell Type	Age[a]	Survival relative to Hippocampal Neurons
Cortex	E18	82%
Septum	E18	59%
Substantia nigra	E18	47%
Striatum	E18	66%
Cerebellum (granule cells)	P8	91%
Dentate gyrus	P4	66%

Cells were plated at 320/mm^2 and grown for 4 days. relative survival was calculated by dividing survival for E18 hippocampal neurons.

[a]E = Embryonic age gestation, P = Postnatal age in days

the medium to prevent glial cell overgrowth. In Neurobasal/B27, glial growth is reduced to less than 0.5% resulting in a nearly pure population of neurons (2). Neurobasal/B27 will be useful in studies of neuronal development, plasticity, electrophysi-ology, gene expression, pharmacology, and neurotoxicity. As with other serum–free media, Neurobasal/B27 should also be useful in studties of growth factors, hormones, cytokines, and other bioactive compounds. In addition, Neurobasal/B27 allows the study of individual isolated neurons for several weeks without a glial cell feeder layer.

REFERENCES

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2. Brewer, G.J., Torricelli, J.R., Evege, E.K., and Price, P.J. (1993) *J. Neurosci. Res.* 35, 567.
3. Bottenstein, J.E. and Sato, G.H. (1976) *Proc. Natl. Acad. Sci.* USA 76, 514.
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7. Wallace, T. and Johnson, E. (1989) *J. Neurosci.* 9, 115.

Product	Cat. No.	Size
Neurobasal™ Medium (1X)	21103-049	500 ml
Neurobasal™ Medium without Phenol Red	12348-017	500 ml
Neurobasal™-A Medium (1X)	10888-022	500 ml
Neurobasal™-A Medium without Phenol Red	12349-015	500 ml
B-27 Supplement (50X)	17504-044	10 ml
B-27 Supplement Minus AO (50X)	10889-038	10 ml
B-27 Supplement Minus Retinoic Acid (50X)	098-0153SA	10 ml
N-2 Supplement (100X)	17502-048	5 ml
G-5 Supplement (100X)	17503-012	1 ml

For complete descriptions, see Chapter 3 in the GIBCO™ 2002 catalog.

Spotlight on BioProduction

Multifaceted, Multifunctional Support for Manufacturers

If your concerns are focused on the long stretch between process development and large-scale manufacturing, this column is for you.

GIBCO™ cell culture products and services are used in product development and large-scale manufacturing by leaders in the biotechnology, pharmaceutical, agricultural, and chemical industries. Applications include the manufacture of biopharmaceuticals, vaccines, cell and gene therapy products, and diagnostics for a wide variety of disease states.

Our purpose is to increase your efficiency and productivity in every way that we can. To that end, we will develop a close relationship with you and your company, providing you with a depth of support that is unparalleled in the industry.

We know the challenges you face as you balance the need for quality with cost considerations. We will work with you to help you select the products and technologies most appropriate for your requirements and goals.

You can count on us to show you ways you can economize without compromise—with new products and processes, and by taking advantage of economies of scale.

To help you decide among options, we can work with you to develop total cycle cost and return-on-investment analyses.

We can configure virtually any of our capabilities to suit your needs. No matter what you manufacture, if cell culture is involved, we can help you be more efficient and productive—by providing you with products, systems, technologies and packaging customized to meet your specific requirements.

Trust Our Team

The GIBCO™ BioProduction team is dedicated to meeting your needs for service, guidance, and support. The team is comprised of seasoned scientists with specific applications expertise who focus on new products and technologies, global support, and field engineering excursions. These team members link to all of our internal cell culture departments including R&D, Regulatory Affairs, Process Development, Logistics, Quality Systems, and New Product Development.

The team includes the following individuals. They welcome your inquiries; please feel free to contact them at 800-955-6288.

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Going Places

We will be on hand at the following events this year. Please join us at our exhibits and presentations.

April 23–28
Analytica
Munich, Germany

May 15–18
Society for Investigative Dermatology
Century Plaza Hotel
Los Angeles, California

May 19–22
The Waterside Conference
Hilton DeSoto Hotel
Savannah, Georgia

May 20–22
American Society for Microbiology (ASM) Salt Palace Convention Center
Salt Lake City, Utah

May 25–28
European Society for Human Genetics (ESHG)
Strasbourg, France

June 5–9
American Society for Gene Therapy (ASGT)
John B. Hynes
Memorial Convention Center
Boston, Massachusetts

June 9–12
Bio 2002
Metro Toronto Convention Center
Toronto, Canada

June 20–23
Association of Genetic Technologists
Hyatt Regency
Cincinnati, Ohio

June 24–26
Raw Materials & Contract Services
Marriott
Salt Lake City, Utah

June 26–29
Congress on *In Vitro* Biology
Disney's Coronado Springs Resort
Orlando, Florida

July 13–17
Federation of European Neurosciences Societies (FENS)
Paris, France

July 20–24
American Society for Virology
University of Kentucky
Lexington, Kentucky

September 30–October 3
Cell and Tissue BioProcessing
The DoubleTree Resort Hotel
Santa Barbara, California

October 2–5
International Genome Sequence & Analysis (GSAC)
Boston Marriott & Sheraton
Boston, Massachusetts

October 15–19
American Society of Human Genetics (ASHG)
Baltimore Convention Center
Baltimore, Maryland

November 2–7
Society for Neuroscience
Orange County Convention Center
Orlando, Florida

November 11–14
Viral Vectors & Vaccines
The Astor Crowne Plaza
New Orleans, Louisiana

December 6–10
American Society For Hematology (ASH)
Convention Center
Philadelphia, Pennsylvania

December 14–18
American Society for Cell Biology (ASCB) Moscone Center
San Francisco, California