Culex ABS
Part I: Introduction to
Automated Blood Sampling

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*At the National AALAS annual meeting (November, 1999) BAS introduced a new class of instrument. The Culex Automated Blood Sampler (Culex ABS) was designed to facilitate research in pharmacokinetics, drug metabolism and toxicology by automating the collection of whole blood from awake and freely-moving rats. This approach eliminated the extensive animal handling and restraints normally associated with manual blood sampling. In fact, rats in this system could roam freely in a cage, with food, water, and no interference in their normal activities **while** blood sampling was underway. A single animal could be used for studies ranging from a few hours to several days. The sampling times, and the blood volume removed at each time point, were programmable by the user. Four animals could be housed in the system with asynchronous sampling protocols.*

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The Culex ABS takes less than 0.75 square meters of floor space in the laboratory, but still houses four rats, each with its own cage and associated blood sampling apparatus. The system collects blood in sealed, refrigerated vials which are transferable to a 96-well plate. Urine is collected in chilled scintillation vials. Feces are collected separately on a stainless steel screen. Animal activity throughout the sampling experiment is recorded as part of the archival record.

More information is available at the **www.culex.net** and in a video tape presentation which may be requested from BAS, or via the feedback form on the web site.

The technology used in the Culex is covered by USA and international patents, both issued and pending[1].

New technologies implemented over the past decade have improved many aspects of drug screening. Organic chemists have accelerated the front end of the process by increasing the production of potential candidates. Analytical chemists have increased the pace at which biological fluids can be analyzed by incorporating automated extraction tools, autosamplers, 96-well plates, and LC/MS/MS into their arsenal. Large scale in vitro screening protocols involving microsomes, hepatocytes or other biomaterials have further aided the process.

The need still remains for data from live animals with functional metabolic systems, or special characteristics (e.g. transgenic rodents). The collection of biological fluids, especially whole blood, is integral to drug metabolism and pharmacokinetics research. This area of new drug research is ripe for improvement. The prevailing methodology requires hand labor, animal restraints, and sampling protocols which extend beyond the normal work day. Evolving animal use and care regulations create other con-

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cerns. Clearly the time has come for a means of automating the collection of biofluids from rats, while keeping animal welfare concerns in mind.

A Starting Point

There have been several reports in the scientific literature[2,3] over the past twenty years outlining alternate ways to remove blood samples from rats. A review of this literature shows that while plenty of energy has been applied to this problem, various shortcomings have limited the practicality of these approaches. For example, most systems do not provide an adequate method of managing the collection lines or tubing, relative to normal movement of a healthy animal. Approaches involving restraining chambers or other devices which prevent the animal from moving will protect catheter lines but often stress the animal to a level that affects the experiment, sometimes to the point of failure. Attempts to hide catheter lines in jackets for long periods eventually fail as the animal wiggles free of the jacket and then chews the line. Various liquid swivel arrangements appear in these papers, but swivels themselves are problematic due to a) materials which initiate clotting b) limited numbers of channels c) cross-channel leakage and contamination, and d) the inconvenience or inability to be adequately clean and sterilize swivels between uses.

Our Approach

As described in the second part of this introduction[4], the Culex evolved from the BAS Raturn System for

F2

The Culex cage has a hinged door for access to the animal during drug administration, and installation. This door can be completely removed, as shown, for the operator's comfort. Since the rat is tethered to a steel wire, escape is unlikely during the short period that the cage is open. The door is closed and locked during normal operation and blood sampling.

Awake Animals, a device which protects multiple lines of tubing, wires, or cables without impeding the normal movement and activity of a healthy animal. The Culex was specifically designed for pharmacokinetics and drug metabolism, with applications for toxicology studies as well. It removes blood painlessly from an awake and freely-moving animal.

Collection without Clotting

With the Culex, the blood does not enter into other devices. Blood leaves the animal, enters the catheter tubing and remains inside tubing of the same chemical composition until it is finally deposited in a sealed, refrigerated sample vial. The design of this system deliberately avoided transfer of blood through disparate materials, such as those found inside liquid swivels. Blood clots form on materials which are foreign to the body through a complex process involving proteins which adhere to the foreign surface and initiate further protein activation and clotting activity. By reducing the number of materials to which the blood is exposed, and using materials which are less likely to activate the thrombogenic proteins, we were able to develop a system in which blood could be removed and returned to the same animal, without clotting.

In addition, the blood is moved through the system by sterile saline containing a low titer of the antithrombogenic agent heparin. The heparinized saline continually washes the tubing to help retard the initiation of clotting in either the tubing set or catheter.

Over time, it is possible that natural clotting mechanisms could overcome these features in the tubing set, since this is what often occurs in various biomaterial implants in the human body - such as stents. To avoid this and maintain the sterility and accuracy of the system, the tubing set was designed to be disposable, with a single use envisioned for each animal.

Why Call It Culex?

The Culex ABS is named after the most common genus of mosquito found in the eastern USA. As a device which removes small amounts of blood painlessly from animals, uses anticoagulant materials to prevent clotting during collection, and is relatively compact, it serves its namesake well. In a departure from the mosquito model, there have been no reports of itching after Culex use.

How It Works

The Culex is a blood sampling robot which is controlled by its own internal computer. The user instructs the robot about the details of each sampling protocol, which is called a "method". A method includes several operating parameters such as the time when a blood collection will take place (relative to the start of an experiment). The volume of blood to be collected (10 to 250 μL), the volume of saline to be added to the blood sample (this choice affects whether the collected blood can be processed into serum or plasma), and the start method (instant or remote). The method details are transferred to the robot via the user interface on a PC with the Windows (95/98) operating system installed.

Once the method instructions are received, the method will begin according to the start technique specified. An instant start begins when the user clicks the RUN key on the computer display. A remote start begins when the user touches the RUN key on the Culex controller keypad. Each start method has advantages. However it is more important to consider when and how the drug will be administered to the animal. There is a "countdown" timer on the front panel of the Culex controller which counts down the time (HH:MM:SS) until the next blood sampling event will occur. For example, a user could program collection of three blood samples and watch the countdown timer to wait until one minute prior to collection of the fourth sample

F3

The Culex sampling process uses heparinized saline as a motive force to transfer blood through the system. This saline can be added to the collection vial to prevent clotting if the final goal is to obtain plasma. If blood serum is the desired result, the saline can be diverted so that only whole blood is deposited in the vial, where it can clot. The process is described in the text of this article. Combinations of the eight events shown here make it possible to obtain either serum, or plasma from the collected blood sample. The sequence will vary according to the desired outcome.

These drawings are not to scale and represent only a schematic.
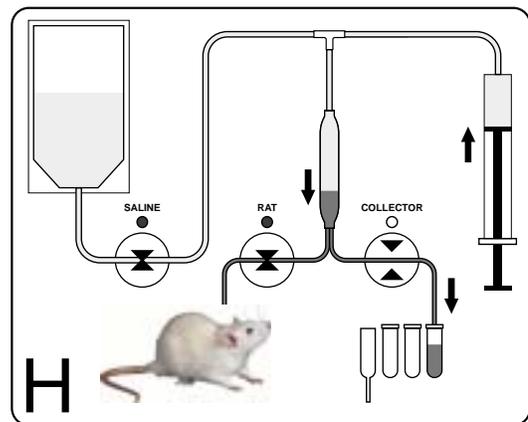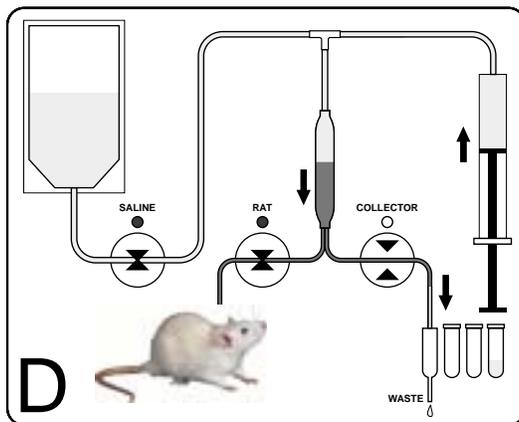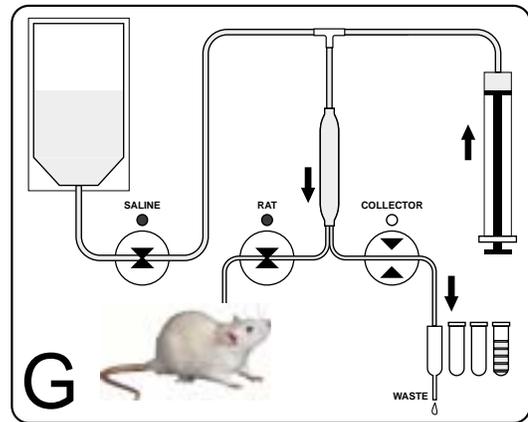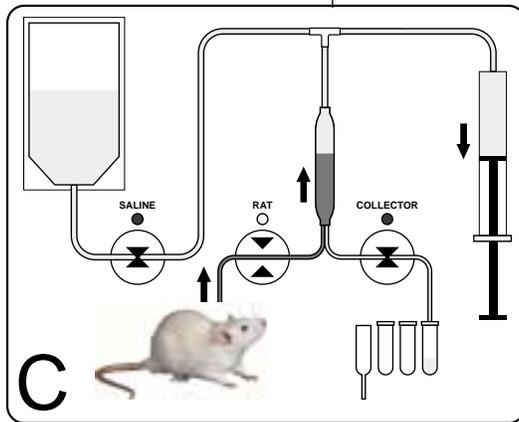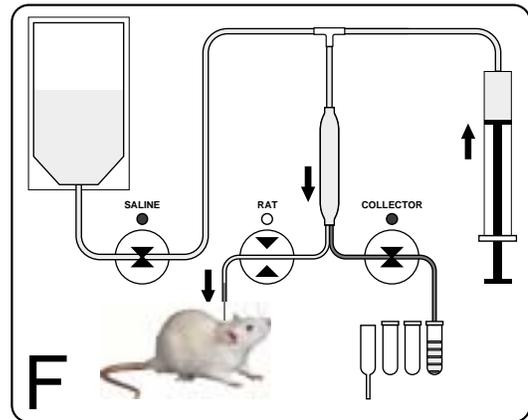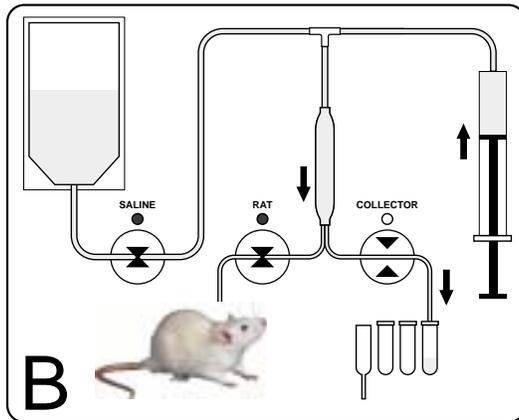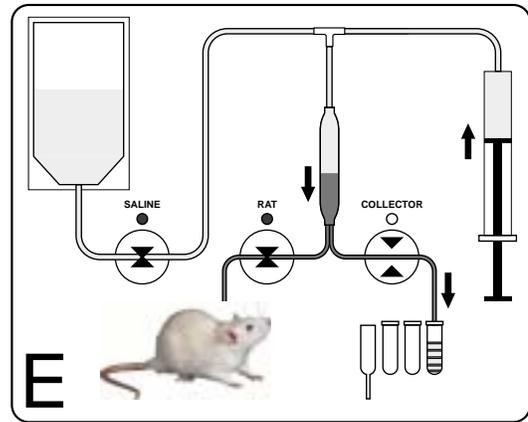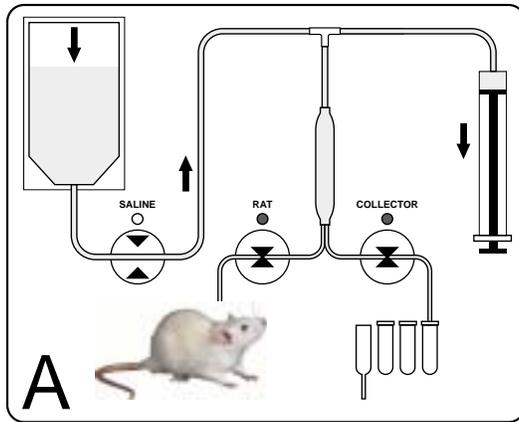
Whole Blood

Saline

Blood diluted with saline

Blood samples are collected into pre-sealed borosilicate glass vials which are refrigerated at a temperature of 3°C. The entire carousel of samples can be removed, as shown, after the experiment and stored. Or, each vial can be removed and transferred to a centrifuge, or to a 96 well plate for centrifugation and processing by an automated system in the analytical laboratory.

before injecting the drug. The fourth sample would then represent the one minute time point in the PK curve. IV injections are much easier to time precisely than gastric lavage which may take more manipulation and patience to administer. Regardless of the method of drug administration, the actual time of the event is time-stamped by pressing the event marker on the Culex front panel when the animal has been dosed.

When the time approaches the start of a programmed blood sampling event, various activities will already have begun. Syringes will have been refilled, collection vials will be moved into position, lines will be cleared and all will be ready for blood transfer.

The best way to appreciate the blood sampling mechanism utilized by the Culex is by viewing either the Flash animation presented on the *www.culex.net* website, or by watching the videotape presentation.This is a dynamic process, with multiple activities occuring simultaneously. The next section attempts to explain the approach by breaking the process into a series of discrete events, but the process is not static, as a first glance at the illustrations (**F3**) might suggest.

The Sampling Process

The Culex ABS was designed to collect whole blood from awake and freely-moving animals. Analytical methods for DMPK normally require the preparation of either plasma or serum from whole blood.

These fluid portions of whole blood are obtained by centrifuging the sample to remove cells, and then harvesting the resulting supernatant. The difference between the two components is the treatment prior to centrifugation. When serum is required, the blood is allowed to clot fully, so that fibrin is removed from the fluid portion, along with the cells. When plasma is needed, an anticoagulant, such as heparin, is added to prevent clotting.

The Culex sampling process uses heparinized saline as a motive force to transfer blood through the system. This saline can be added to the collection vial to prevent clotting if the final goal is to obtain plasma. If blood serum is the desired result, the saline can be diverted so that only whole blood is deposited in the vial, where it can clot. As illustrated in **F3**, it is possible to use the Culex to prepare a blood sample suitable for either serum, or plasma, through an *automated* sequence of events.

The process begins with step A, when the syringe is refilled with sterile, heparinized saline. Only the valve at the *saline* position is opened, while the syringe plunger is withdrawn. If the blood will be processed into plasma, then step B is added and a user-specified volume of saline is dispensed into the empty collection vial when the syringe plunger is advanced and the valve at the *collector* position is opened while all other valves are closed. In step C, the valve at the *rat* position is opened, and the syringe is again withdrawn. This sequence pulls blood from an intravenous catheter in the animal and into the tubing set on the Culex. Position D now diverts the collected blood into a different line when the *collector* valve is opened and the syringe action is reversed. Saline already present in the collector line precedes the blood down this pathway. This saline is dumped to a waste position on the fraction collector, along with the saline/blood mixture at the interface of these two fluids. The next step is represented by either E, if plasma will be processed, or H, if serum will be processed. In both cases, whole and undiluted blood is sent to the collection vial. In E, the vial was already partially filled with heparinized saline, so the blood will not clot. In H, the blood is sent to an empty vial where it will clot in a matter of minutes. Steps F and G represent the cleanup process at the end of each sample. In F, any blood remaining in the tubing set, and catheter line will be returned to the animal, along with a volume of ster-

Intravenous drug infusions may be conducted while the blood sampling experiment is underway. In this illustration, a BAS Baby Bee syringe drive is outfitted with a sterile, disposable syringe and connected to a catheter in a separate vein (e.g. jugular for blood sampling, femoral for drug infusions). A BAS catheter can be used for either sampling or infusions, using the same zero dead volume connection approach.
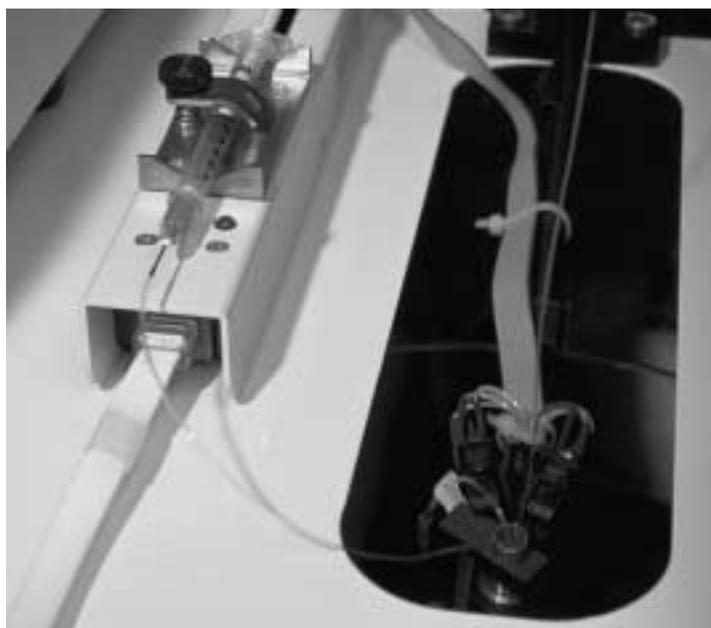
The protocol for the blood sampling experiment is defined by the user in a window such as the one shown. Time intervals can be selected according to user preference and can begin with a time zero (0) sample which is taken as soon as the method is started. The next sample must be at least five minutes later. The user can choose to dilute the sample with heparinized saline (for plasma), or collect whole, undiluted blood which will then clot in the vial. The software records all dilution factors so that this information can be printed (on paper or in an electronic text file) for transfer to the analytical chemist processing the samples. The software tallies all of the blood that will be removed from the animal during blood sampling, including blood transferred to the sample vials and the small amount lost during the sampling process. When a new method is saved, a password will be requested by the software. Only the password holder will be able to make changes to this method in the future, although the sampling protocol will be visible to all users, and available for them to employ. The method ID is indelibly stamped on the method and will change if any modifications are made to the file by the password holder.

ile saline equal to the volume of blood taken from the animal by the collection process. This enables the animal to maintain fluid balance. The heparin in the saline is metabolized by the animal. In step G, any blood remaining in the collector line is flushed to waste. The system is now ready for the next blood sampling event which begins again with step A.

A "plasma" collection would therefore include the sequence of steps A, B, C., D, E, F and G. A "serum" collection would include A, C, D, H, F and G. Note that the serum version of step C would have no saline in the collection vial. Remember that in both cases, the vials contain whole blood which is then centrifuged offline to obtain either plasma, or serum.

24/7 Operation

The designers of the Culex anticipated its operation around the clock. To preserve the samples, blood is collected into cold vials, which are automatically maintained at 3°C as long as the system has power. Individual vials can be removed from the fraction collector without disrupting normal system operation. Or, the entire carousel of vials can be removed after the experiment is completed, as shown in **F4**, and transferred to a refrigerator or the analytical laboratory. Vials are sealed with plastic

snap caps prior to installation in the fraction collector. Caps retard evaporative loss of the sample and keep out dust during collection of blood. A stainless steel needle on the fraction collector pierces the thin plastic septum on each cap when the blood is collected.

The caging system in the Culex provides food and water for the animal, which roams freely in the cage without damaging the catheter and infusion lines. Underneath the cage, urine is collected and separated from fecal pellets. Urine is chilled in a scintillation vial, maintained at ≤ 4°C for up to 16 hours. These features are described in more detail in Part II of this series.

Drug Administration

New drug candidates are administered to animals through a variety of methods, such as injections (IP, IM, subcutaneous, subdermal), gastric lavage, diet, and intravenous infusion. The Culex has several features to accommodate these techniques.

For feed-based formulations, the stainless steel food bin is used. The bin is easy to weigh and holds enough food for 1-3 days, depending on the animal's size. It can be added or removed from the cage, without tools, by sliding into a bracket on the cage wall. Animals will usually feed directly from this bin with excess food falling back into the same bin.

For intravenous infusions, a BAS Baby Bee syringe drive and sterile, disposable syringe can be placed directly adjacent to the counterbalanced arm over each cage. If the animal has been implanted with another BAS catheter in a second vein, the connection to the syringe can be made with a zero dead volume connector. In the first release of the Culex software, drug infusions are not programmable within the method. Instead, infusion start/stop times are controlled through a separate syringe pump controller on the stand.

Methods requiring animal handling, such as gastric lavage or injections, can be accomplished while the animal is installed in the Culex cage and connected to the blood sampling apparatus. The tether line keeps the animal from escaping and a large door in the cage provides room to work. The door can also be lifted off its hinges if even more work space is needed.

Basic Software Features

The features of the Culex control software are best appreciated by reviewing the manual which is available for review on the *www.culex.net* web site. We will address only a few of them in the space available to us in this introductory article.

The software runs under the Windows operating system (95/98)

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from a laptop PC mounted on the Culex. It controls aynchronous blood sampling experiments for four animals, each of which can also be running an independent method.

When the system is in use, a display screen shows the status of each experiment. You can determine when the experiment began, which method is being used, when the next blood sampling event will occur, when the experiment is scheduled to end, what the Culex is doing at that moment, and how active the animal has been since the start of the experiment.

A data log file is created for each of the experiments running on the Culex. The log file provides a live stream of raw data being captured by the computer and is a useful diagnostic tool for the experienced user. Once the experiment is complete, a variety of reports can be selected by the user, each of which will access different parts of this raw data. Each report includes a header with the calendar date/clock time of the experiment, along with observations, events and other descriptive information entered by the user, a unique method ID, and the method filename. One report, which should accompany the samples to the

analytical lab, includes the dilution factor (if any) for each sample.

Tabs on the screen enable the user to select information for each animal station, identified as Culex A, B, C or D. The identity of each Culex station can be determined by looking at the LCD display on each of the four Culex Controllers.

Method Integrity

Creation of a blood sampling method for the Culex is a simple matter. Editing an existing method is more complicated.

F6 illustrates the window used to create/edit a method. After choosing the manner in which a blood sampling experiment will start, as previously described, the user selects the type of BAS catheter implanted in the animal. The user can then create each line in the protocol through a combination of the sample time, blood volume and saline volume. Clicking the ADD key will insert the new line at the cursor position. The time points listed are in relative time. That means it is relative to the start of the experiment. In the example shown, samples were taken immediately (0 minutes) and then every five

minutes thereafter for several hours. Sample times must always be entered in minutes, but the method can also display the times as D:H:M when D = days, H = hours and M = minutes after initiation. MODIFY and DELETE keys are used for line by line corrections.

Once the method is complete, a password is requested by the system as the file is saved. The screen will not clear at this point. Instead, a new line called METHOD ID will appear along with a string of numbers. The METHOD ID number is a unique identifier for the method file. It will appear on all reports to verify which method was used for the blood sampling experiment. Any person using the Culex can go to the list of method filenames and select a method (*filename.clx*). The METHOD ID will appear on the screen and on all logs and reports associated with the experiment.

If one of the users of the Culex attempts to change the method file, he/she will be prompted to provide a password. No changes will be made to the file without this password. If the password is provided and the file is saved under the same filename (e.g. candice.clx), the method ID

will change automatically. Any subsequent use of the candice.clx method will exhibit a new ID, indicating that changes were made to the original method.

If a user replicates all of the lines in a method and saves it as a new method, the Method ID will be different again.

There is no way to modify a Method ID once it is assigned, and no way to keep the old ID if a method is changed in any way. This feature provides a measure of security for the principal investigator who can verify that the correct method was used for the sampling experiment by reviewing one point of information.

Time Stamping

Certain events in a blood sampling experiment have special significance. One of these is the time that the drug was administered to the animal. The Culex includes a means of time-stamping the data log for each experiment. When the EVENT button on the front panel of each controller is pressed, it time-stamps the data log and permits the user to go to the keyboard and enter text describing the significance of that notation. Events are part of the permanent record for the experiment and appear in all printed reports.

Conclusion

The Culex ABS is a robotic system designed to accelerate the pace of drug discovery research by automating a process that is currently conducted by hand. Serial blood sampling is conducted in a single animal. Technicians can generate more blood samples through round the clock operation, and the ability to manage more animals at one time. The avoidance of animal handling improves reliability of catheters and consistency of data. The Culex ABS was also designed to maintain animal health by minimizing stress, maintaining fluid balance, providing food and drink, and offering freedom of movement.

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