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GMP Compliance for the Production of the Monoclonal Antibody CB.Hep-1 Used as Biological Reagent

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Abstract

CB.Hep-1 is a mouse monoclonal antibody (MAb) secreted by the hybridoma 48/1/5/4, specific for the “a” determinant of the Hepatitis B surface antigen (HBsAg). This MAb is routinely used for the immunopurification of recombinant Hepatitis B surface Antigen (rHBsAg) for vaccine purposes. Considering the application of this MAb and the issues raised by regulatory agencies for the manufacture of biological products, a documentation system and methods to control the MAb production process were established.

Starting in 1991, the department responsible for immunosorbents CB.Hep-1 production has followed Good Manufacturing Practices (GMPs) regulations by establishing a complete documentation system, in which the main objective has been to guarantee stable and solid production processes for the preparation of biopharmaceutical products.

The principle recommendation in this paper is the application of the MAb as biological reagent in the drug substance manufacturing process where the MAb is used to purify the drug substance.

Several issues that may be useful in developing a regulatory strategy for monoclonal antibodies used as reagents are discussed.

Introduction

A Good Manufacturing Practice (GMP) system constitutes an associated group of norms and activities destined to guarantee that every product meets and retains the characteristics of design required for its use. The GMPs also minimize unforeseen risks that may occur during the screening of the final product.1 These norms establish requirements for the production of drugs for human use on both medium and large scale manufacture. Conformance to the GMPs is basic to state of the art quality systems.

The documentation system is the essential part of any quality system; therefore, it should be based on the GMPs and must comply with all relevant aspects for the production and commercialization of the products. During the production, control, and administration of any pharmaceutical product, special care must be taken because of the many chemical biological substances, live organisms (cells), and various production processes that could be involved. These variables demand that any documentation system provide all possible information about the history of the product from the very beginning of the

production process and must fulfill all regulatory requirements for these kinds of products.2

The technique of Köhler and Milstein,3 involves immortalizing antibody-producing plasma cells from an immunized mouse by fusion with myeloma cells (a plasma cell tumor line). The resulting hybrid cells or hybridomas can be maintained *in vitro* or *in vivo* to continuously secrete large quantities of MAb with a defined specificity and can be purified and used in a variety of applications.4, 5

The main characteristic of these products is that they are produced by mammalian cells in culture or are taken directly from an animal. In both cases, the starting material is capable of harboring adventitious agents - generally viruses that can result in significant harm to humans.6 Process validation is an important tool for the evaluation of biotherapeutic products, such as MAbs obtained from mice.

In this paper we introduce and apply applicable GMP elements and a documentation system that supports process consistency in the production of immunosorbent CB.Hep-1.

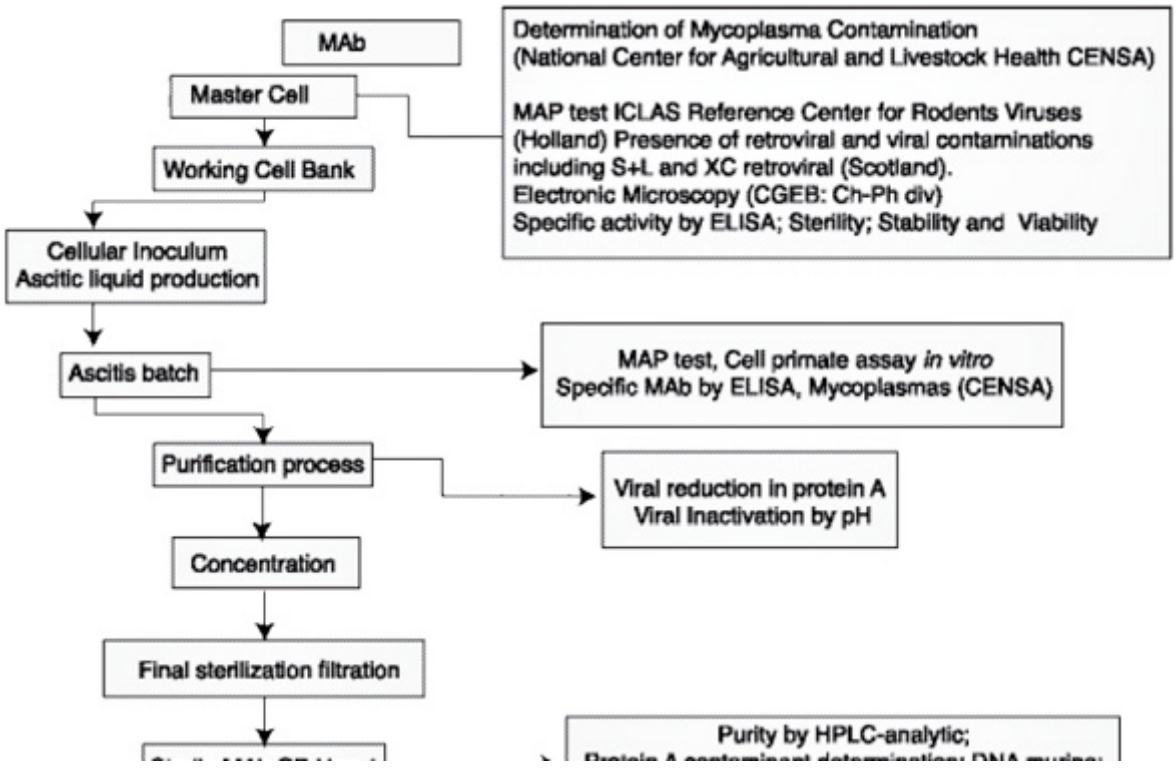
CB.Hep-1 Monoclonal Antibody (Anti-HBsAG) Immunoaffinity Matrix

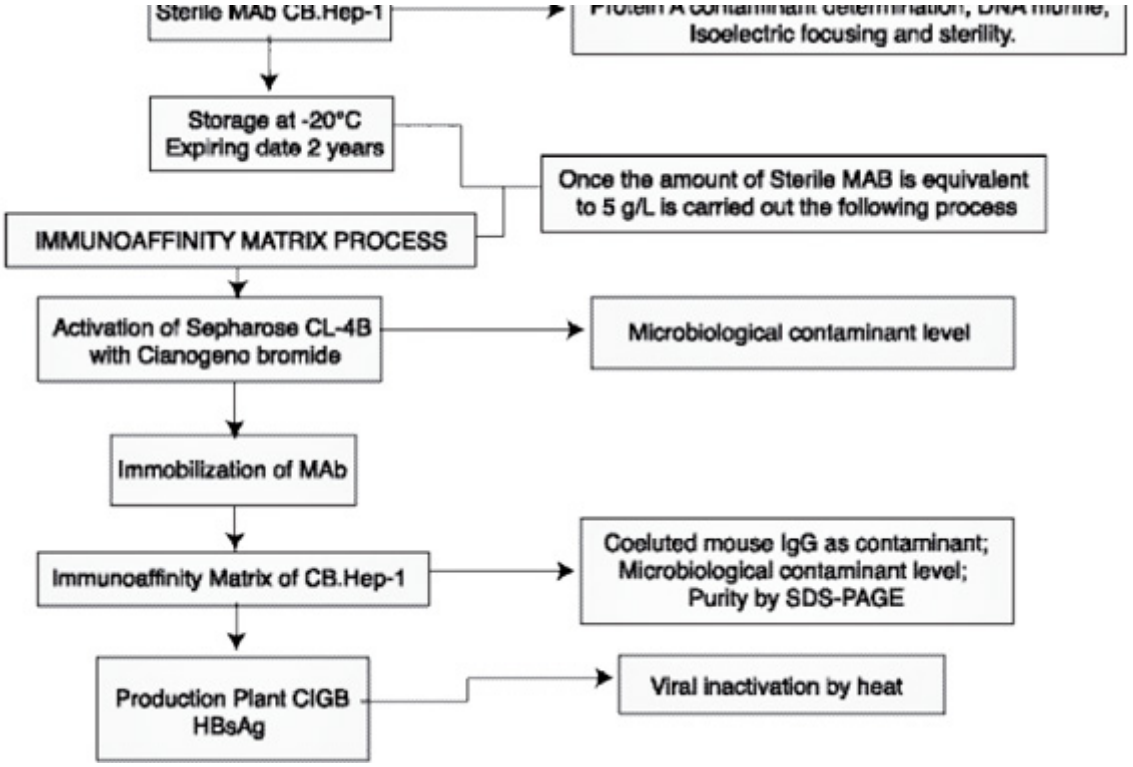
The production process of MAb CB.Hep-1 includes four main steps:

1. Conformation of the cell banks and inoculum
2. Ascites production
3. Immunoglobulin G (IgG) purification
4. Immobilization on Sepharose CL 4B7

In every step, several unitary operations, processes, and quality controls are carried out. All data generated during this process has been analyzed using the Microsoft® Excel software version 7.0. (See *Figure 1*)

Figure 1: Flowchart of the Immunosorbent CB.Hep-1 Production and Controls.





Inoculum Preparation

Inoculum preparation includes maintaining the Master and Working Cell Banks (MCB/MWCB) and growing the suspended or stationary methods in spinner flasks. Each production batch is originated from a fresh ampoule of the seed (e.g., working cell bank). The maximum permissible number of serial passages in culture during normal production is defined and restricted. Justification of this limit should include information concerning the yield of monoclonal antibodies and the stability of the hybridoma.

<pSubsequently, 106 hybridoma cells are inoculated intraperitoneally in each mineral oil primed BALB/c mice.8 Media and buffer preparation is performed manually with powders in mixing tanks.Purification consists of several chromatography gel filtration cycles in Sephadex G-25 coarse (Amershan- Pharmacia, Uppsala, Sweden) Protein an affinity.9 After this chromatography, incubation in 0.1 molar (M) citric acid pH 3.0 for an hour at 4°C was carried out. The ultra filtration and sterile filtration steps complete the basic diagram of the purification process.10 The purified antibody is stored at 4°C for further use as immunoligand to purify the rHBsAg. The final product of the purification process is controlled and released by the Quality Control Laboratory. (See *Figure 2*.)

Immunoaffinity Matrix

The purified antibodies are bound to the Sepharose CL-4B (Pharmacia) activated with cyanogen bromide, as recommended by the manufacturer (Pharmacia-LK B, 1993). Before release of the columns, they are submitted to a test to verify their functional performance. (See *Figure 3*.)

Bio-safety of the Process

In order to guarantee the quality and safety of the immunogel CB.Hep-1, the production process is submitted to a strict control of the biological material involved in the process, which is based on the following strategy:

1. Serologic control of the colonies of mice used to produce the ascitic fluid CB.Hep-1
2. Certification of the fetal bovine serum
3. Certification of the master cell bank
4. Virological control of the ascites
5. Viral validation of the purification process.

Alemán R and Valdez R, two of our principal investigators in two separate works, have published these strategies.10, 11

Documentation

Documentation is the key to a consistent, controlled process according to Vasilevsky.12 The documentation system is integrated with different documents approved by the Quality Assurance (QA) Department following GMP requirements. These include: Standard Operation Procedures (SOP), quality specifications, batch production records, the legal license to produce, as well as other documents such as, flowcharts, cleaning standards, calibration and equipment maintenance records, etc.

Figure 2: Inspection Points

Test	Method	Acceptance Limit
Concentration of the specific MAb	ELISA	Identified, Quantified
Total protein concentration	LOWRY	Inferior limit: 4 mg/ml Superior limit: 17 mg/ml
Specific activity by relation IgG by ELISA x 100 Protein by LOWRY		? 70%
Contaminant DNA	Hybridization	? 10 pg / mg total protein
Sterility	WHO T.R.S. No.530, 1973	Passes the test
Protein A contaminant level in MAb	ELISA	? 10 ppm
MAb isoelectric point determination	Electrofocusing	Presence of the 4 majority bands from the 8 originally monoclonal ones
Purity Level By		
SDS-PAGE	SDS-PAGE	? 90 %
Molecular size exclusion HPLC*	HPLC	? 95 % (area under the main peak)

* High Performance Liquid Chromatography

Figure 3: Quality Specification of the Immunoaffinity Matrix

Test	Method	Acceptance Limit
Coeluted mouse IgG as contaminant SDS-PAGE Microbiology Control	ELISA SDS-PAGE	< 3 ng IgG/?g rHBsAg ? 80% ? 23 nmp of microorganism/mL

In addition, organization and personnel-related detail is included, such as: organization charts of the Production Department, descriptions of all the positions and responsibilities, as well as control lines and subordination. Behavior, hygiene, manners, and the training of personnel are described in detail in the organization and personnel data.

Results and Discussion

Guidelines for the development of MAbs are published and periodically updated. (See *Figure 5*.) These guidelines serve as blueprints for the manufacture, safety, and efficacy testing of MAbs. Since biotechnology and the development of MAbs are rapidly evolving fields, the information contained in these guidelines may become obsolete or be subject to rapid change as new and more significant information becomes available.

In the following table, and keeping in mind the guidelines issued by the World Health Organization (WHO) and FDA, we can see the guidelines for MAbs used as reagents and parenteral therapeutics. The regulations on the use of monoclonal antibodies are the same in both cases; its demanding features are the same. *(See Figure 5)*.

Figure 4: Standard Operating Procedures.

List of the SOPs that describe the main operations of the production of anti-HBsAg monoclonal antibody and the immunoaffinity matrix.

Code	Title
SOP 2.22.005.92	Culture medium preparation and solutions at the cell culture step
SOP 2.22.087.92	Master Cell Bank preparation for MAbs production
SOP 2.22.088.92	Working Cell Bank preparation for MAbs production
SOP 2.22.164.94	Precipitation of MAbs for pharmaceutical purposes
SOP 2.22.165.94	Standard operating procedures for performing the Sephadex G-25 chromatography
SOP 2.22.167.94	IgG antibody purification through Sepharose Fast Flow Protein A chromatography
SOP 2.22.006.92	Filtration of supplemented culture medium, process solutions, and purified MAbs
SOP 2.22.199.95	MAbs immobilization using a stirred tank
SOP 2.22.219.99	Concentration of MAbs through DC-10 concentrator equipment

Figure 5 The Main Guides, Codes, and Regulations for the Production of MAbs

1. U.S. Department of Health and Human Services Food and Drug Administration (FDA). “Points to consider in the manufacture of in vitro Monoclonal Antibody Products which are identified as Biological Products.” June 1983.
2. U.S. Department of Health and Human Services Food and Drug Administration (FDA). “Points to consider in the manufacture of Monoclonal Antibody Products for Human use.” July 1983. Only apply to monoclonal obtained from hybridomas of murine-murine origin.
3. U.S. Department of Health and Human Services Food and Drug Administration (FDA). Center for Biologics Evaluation and Research (CBER). “Points to consider in the manufacture and testing of Monoclonal Antibody Products for Human use.” February 28, 1997.
4. FDA’s Policy Statement Concerning Cooperative Manufacturing Arrangements for Licensed Biologics. Nov 1992
5. “Points to consider in the manufacture and testing of Monoclonal Antibody Products for Human Use.” Aug 1994
6. U.S Department of Health and Human Services Food and Drug Administration. Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER). Guidance for Industry Monoclonal Antibodies Used as Reagents in Drug Manufacturing. May 2001

WHO Technical Report Series 822

This document applies to murine and human monoclonal antibodies for use in humans, including *in vivo* diagnosis and *ex vivo* (extra corporeal) treatment. It is recommended that monoclonal antibodies intended for use in the preparation of biological products in humans should also meet these requirements, except for those relating to the final product. (This document is not concerned with the production of monoclonal antibodies by recombinant DNA techniques, such as “humanized” antibodies, nor with monoclonal antibodies to be used for in vitro diagnostic purposes.)

Food and Drug Administration (FDA) 1997

The FDA documents apply to MAb used as therapeutic or in vivo diagnostic agents, as well as to ancillary products, i.e.: MAb used in the manufacture of other products for in vivo use. The latter include MAb that are used alone or in conjunction with devices, for example, for ex vivo purging of cells to remove immune or tumor cells, for ex vivo collection (e.g., hematopoietic cells), or for purification of other products intended for in vivo administration. Generally, these MAb should meet the same criteria for safety and freedom from adventitious agents as MAb intended for direct administration to patients.

In Figure 6, we can see that regulations covering the use of monoclonal antibodies are the same in both cases; its demanding features are the same. In our case, we also took into account the regulations as have been previously noted.

However, when analyzing the higher cost of each assay made in the process of a monoclonal antibody and following all the rest of the required analyses, we arrive at the conclusion that it is quite high. We could ask: “Why it is necessary to maintain the same regulations for MAb use as a reagent to purify biologicals?” The recommendations for characterization and testing of MAbs used as parenterals are, of necessity, very stringent. Not all of them would be applicable to MAbs that are used as reagents in drug manufacturing.

To provide an adequate description of the manufacturing technology used in the production of the MAb; to ensure satisfactory performance of the reagent during the process; and to assess its potential impact on the biological safety, quality, and purity; the production of MAbs includes the following phases:

1. Development and characterization of the cell line
2. Establishment of the cell banks
3. Production in animals (ascites fluid)
4. MAb purification
5. MAb linked to Sepharose CL-4B

The basic elements of compliance include: written SOPs, flowcharts, the immunoaffinity matrix, and QC and PC specifications. The written SOPs describe each operation. See Figure 4 where some of the SOPs used in our process, including, approval authority, change control, history files, and standard formats, are listed.13 The flowchart for the production of anti-HBsAg monoclonal antibody and the immunoaffinity matrix is shown in Figure 1. Here, the QC, Quality Control, and the PC, Process Control, stand for the controls that are carried out in every step.

Although experts expound that in the future all the MAbs can be delivered and certificated according to existing regulations, we consider that the regulations for the monoclonal antibodies used as reagents could resemble the following:

Development and Characterization of the Cell Line

This information is important because it will determine, in part, what adventitious agents tests should be performed in those cases where extensive testing may be necessary. (The suppliers certify the fetal bovine serum as free of bovine viruses, Bovine Spongiform Encephalopathy (BSE), and mycoplasmas.)

- Other characterization, such as subclass and molecular weight determination, should be performed early so that appropriate identity tests can be developed for production lots.
- Major concerns in cell bank establishment include verification of the identity and clonality of the banked cells and acceptable degrees of freedom from adventitious agents.

In our case, CB.Hep-1 MCB is free from adventitious agents and the MAb characterization corresponds to the MAb secreted by the original cell line; therefore, it can be used in the rHBsAg purification process. Aleman, et al. 2000, publishes these results.11

Production in Animals (Ascites fluid)

In general, steps should be taken to prevent or control contamination (e.g., virus, bacteria, fungi, mycoplasma) introduced during the production process. Recommended characterization of the MAb production process, again, will depend on the subsequent downstream processing of the drug substance.

Figure 6: Comparison of Regulations for Monoclonal Antibodies

MAb Used as Parenteral Therapeutics and as Reagents	
Master Cell Bank (MCB)	• Identity test for the product • Perform tests for bacterial, fungi, and mycoplasmas • Perform tests for viruses (MAP, S+L-), and XC tests • Perform tests for undesired immunological reactions
Master Working Cell Bank (MWCB)	Complete the same full testing regimen as MCB (When the MWCB is derived from the same MCB, it may be omitted.)
Production Processes	
Mouse Ascitic Fluid	Mice shall be free from the viruses List 1, and if found to be contaminated with viruses List 2, the final product may be accepted only if the purification process is shown to eliminate the infecting virus.

Purification Process	• Validate procedures for removing contaminating cellular DNA • Determine the removal or inactivation of these test viruses • Validate procedures for removing impurities
Production Control	
Bulk Harvest	Every bulk harvest shall be tested for freedom from bacterial, fungal, and mycoplasmal contamination; it shall be tested on primate cell lines and be shown to be free of detectable viruses.
Purified Bulk	• Shall pass the tests for absence of bacterial and fungal contamination. • Test for components other than those secreted by the cell line or the hybridoma. • Test for residual DNA. • Test for contaminating viruses (if bulk harvest from mouse ascitic fluid was contaminated with viruses from list 2 (Appendix 1 822)
Final Bulk Final Product	• Test for identity (isotype composition and for its immunological reactivity with the target antigen • Test for bacteria and fungi (each final bulk) • Test for purity: test for distribution of molecular size Isoelectricfocusing • SDS-PAGE • Test for protein content Test for identity, isotype composition, and immunological reactivity with the target antigen.

Isolation and Purification

For reagents, the predominant concern is whether the MAb purity is sufficient to prevent the carryover of in-process contaminants to the drug substance or product. Consistent production of a reasonably pure reagent should be demonstrated to assure adequate and uninterrupted performance in the production of the drug substance. Downward trends of the MAb lots in purity, integrity, and binding properties have been thoroughly investigated. Chemical impurities originated from buffers, columns, and media, as well as host cell protein, should be monitored and minimized to avoid contamination of the bulk drug substance.

Our purification process was designed taking into consideration a combination of two main principles: saline precipitation to reduce ascites volume and Protein A affinity chromatography. We also introduced into this process, incubation of the IgG for 1 hour at low pH to increase the viral safety of the process as well as several filtration steps to get the required sterility.14

The conditions used in this purification process provided a purity level in the purified MAb higher than the 95% measured by SDS-PAGE and HPL C procedures. In this work, the homogeneity and identity of the purified MAbs were checked by isoelectrofocusing, isotyping, amino acid light-chain composition, and ELISA. The isoelectrocfocusing pattern resulted in the presence of four majorities bands for a total of eight bands, which corresponded with an isoelectric point in the range of 6.75 to 7.04 (data not shown).

Several toxic effects have been associated with the presence of Staphylococcal Protein A.15 The Protein A leakage was determined by means of a specific ELISA, which permitted quantification with high sensitivity and without unspecific reactions to the levels of Protein A in pure samples of the IgG. The level of this contaminant was less than 10 ppm for all batches.16 It represents a very low amount of Protein A and it is not a problem at all because it is drastically reduced during the immobilization of the MAb and also during the antigen downstream process, where the Protein A level was undetectable.

Due to its oncogenic properties, mouse DNA level is an important point to consider. The regulatory agencies are very strict in this area. Today, the acceptable level is less than 100 pg of residual cellular DNA per human dose. One of the advantages of the production from ascitic fluid is the relatively low DNA level in the downstream starting material. In our process, the mouse

DNA present in the purified CB.Hep-1 did not exceed 7 pg/mgIgG.

MAb Linked to Sepharose CL-4B

Tests and acceptance criteria for the monoclonal antibody linked to a solid support include the following:

- Appearance
- Surface density of MAb (milligrams of MAb per gram of resin)
- Specific binding capacity
- Integrity of solid support

With our process, we obtain the following results: purity on the rHBsAg eluted higher than 85%,17 which demonstrated the high purification power and the consistency of this immunochromatographic step. Another aspect of notable importance for the purification of molecules for human use is the level of antibody leakage of the antigen from the matrix. The MAb present in the final pharmaceutical preparation could have several potential negative effects. One could be stimulation of undesired immune responses (HAMA response) and neutralization of the antigen. In this case, the ligand leakage from the matrixes did not exceed 0.005% of the rHBsAg eluted. This represents a very low amount of IgG, especially when we consider that this is the first chromatographic step of the rHBsAg downstream process and that the dose utilized with other mouse MAb is very low.

Saving all of the crucial points mentioned above, the problem resides now in potential viral contamination. In this sense there have been some negative experiences in the biopharmaceutical industry in the last years,18 that is why several controls have been recommended by different guidelines. The MAP test is another recommended assay for determining adventitious agents. We have monitored the MCB, the ascites, and the purified antibodies for several years during which time no viruses have been found.11

The validation of virus removal may be performed during one of the steps in the reagent manufacture, or during one of the steps to the reagent preparation, such as linking the reagent to a column. 2; 14 Validation study provides a high level of assurance that the final product will be free of contaminants. These studies should be done spiking the chromatographic steps with high titer infectious viruses. Several model viruses were used to challenge this chromatographic process. The purification process showed a high clearance factor of all viruses studied.

Conclusion

We have demonstrated that the manufacturing process can consistently produce reasonably pure and active MAb reagent. Since our final product is the inmunogel that constitutes a biological reagent inside the process of purification of the surface antigen of Hepatitis B, its characterization and consistency have been efficiently demonstrated. Quality control tests are carried out routinely on each batch of purified bulk product according to the Guide to GMP.

The positive results obtained during inspections carried out by our regulatory entity, Center for State Control of Drug Quality (CECMED), and the recent WHO inspection demonstrate that we have achieved a product of quality that fulfills the GMP. By keeping in mind all these requirements and by applying GMPs we have achieved the manufacture of a quality antibody for human use.

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