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FULL PAPER

Comparison of two molecular assays for detection of cytomegalovirus DNA in whole blood and plasma samples from transplant recipients

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SUMMARY

In immunosuppressed patients, pre-emptive therapy and a strict follow-up of CMV infection are the standard of care for the prevention of CMV disease. Several real-time PCR assays for CMV DNA quantification on whole blood (WB) and plasma (PL) are commercially available. This study compared and correlated CMV viral loads obtained by the Cobas AmpliPrep/Cobas TaqMan (CAP/CTM) platform on plasma specimens with those obtained on corresponding whole blood specimens by the real-time PCR assay (ELITe MGB-CMV) in 185 sequential samples from 41 immunosuppressed patients. Correlation between the two assays was good. Kinetics of CMV DNA within the same patient was similar, but PL viral load was constantly 1 log lower than WB. In patients under antiviral therapy, low level of CMV DNA persisted in WB, while it was absent in PL. The good correlation between CMV DNA detected on both PL and WB supports the reliability of the two matrices for viral monitoring and the therapeutic management of CMV infection. Nevertheless, due to significant quantification differences between PL and WB CMV DNA, the same biological specimen should be used for a sequential and reliable follow-up of patients at high risk of CMV infection.

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INTRODUCTION

Cytomegalovirus (CMV) infection in immunosuppressed patients, has been associated with systemic and organ diseases, such as pneumonia, hepatitis, gastroenteritis and encephalitis (Paya *et al.,* 1993; Falagas *et al.,* 1997; Krause *et al.,* 1997; Torok-Storb *et al.,* 1997; Zaia *et al.,* 1997; Boeckh *et al.,* 2003). Over the past decade, strategies based on either prophylaxis or pre-emptive antiviral therapy have been successfully used for the prevention of CMV disease in these settings. A major drawback of prophylaxis is the late-onset of CMV disease that occurs in up to 29% of solid organ transplanted (SOT) recipients and is reported in several studies (Humar and Snydman, 2009; Kotton *et al.,* 2010; Gerna *et al.,* 2011a). Pre-emptive treatment of CMV infection is based on the strict virological monitoring of viral replication, for timely antiviral treatment to prevent disease progression. Once viral replication reaches a certain threshold associated with a high chance of developing CMV disease, only high risk patients start anti-CMV medication, thereby avoiding the unnecessary treatment of patients at low risk of disease progression (Boeckh and Boivin, 1998; Boeckh and Ljungman, 2009; Emery *et al.,* 2013; Solano *et al.,* 2013). The efficacy of pre-emptive therapy relies on accurate and sensitive laboratory tests to monitor CMV infection and these are also related to the blood compartment tested (e.g. plasma vs. whole blood).

New molecular technologies based on the quantitative real-time Polymerase Chain Reaction (PCR) have largely replaced conventional PCR and non-PCR methods (e.g. cell culture and pp65 antigenemia), with very satisfactory clinical results (Emery *et al.,* 2000; Tanaka *et al.,* 2000; Yun *et al.,* 2000; Gault *et al.,* 2001; Griscelli *et al.,* 2001; Satou *et al.,* 2001; Gimeno *et al.,* 2008; Boeckh and Ljungman, 2009; Emery *et al.,* 2013; Solano *et al.,* 2013). Currently, there is no universally acceptable viral load threshold for starting pre-emptive anti-CMV therapy. This is related to broad differences in PCR assays accounting for the level of standardization against now available international CMV standards and the appropriate specimen tested. Viral load thresholds to start pre-emptive treatment have been proposed and clinically validated in some settings (e.g. SOT and hematologic patients), but they all refer to a specific biologic matrix (Emery *et al.,* 2000; Stachel *et al.,* 2008; Boeckh and Ljungman, 2009; Gerna *et al.,* 2011a, 2011b; Green *et al.,* 2012; Waggoner *et al.,* 2012). Recently, the first WHO international standard for CMV quantitative nucleic acid testing became available, improving interassay agreement (Fryer *et al.,* 2010). There are several com-

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mercially available real-time PCR assays for CMV DNA quantification on either whole blood (WB) or plasma (PL), with different sensitivity and reproducibility. The majority of these assays are completely automated from nucleic acid extraction to the final real-time PCR result, allowing fewer time-consuming manipulations and a better standardization of the entire procedure. Both PL and WB provide diagnostic and prognostic information on CMV infection. Although WB is considered the most appropriate compartment for CMV-DNA quantification, reflecting both cell-associated and plasma-free virus, PL is an easier biological matrix for sample processing and result standardization than WB (Boeckh *et al.,* 2004).

This study retrospectively evaluated the performances of the novel fully automated COBAS(®) AmpliPrep/COBAS(®) TaqMan(®) CMV (CAP/CTM; Roche Diagnostics, Branchburg, NJ, USA) integrated platform developed and validated against the first WHO CMV standard, for the detection of CMV DNA on plasma specimens from a study group of immunosuppressed patients (Kerschner *et al.,* 2011; Cardenoso *et al.,* 2013; Hirsch *et al.,* 2013; Pritt *et al.,* 2013; Mannonen *et al.,* 2014). PL results were compared with those obtained from the corresponding WB specimen in the same series of patients prospectively tested on WB during the follow-up with a standard real-time PCR assay (CMV ELITe MGB™ kit, Elitech Group, Italy) integrated with the QIAsymphony DNA extraction (Qiagen Italia, Milan, Italy) (Costa *et al.,* 2014). The detection of active CMV infection and viral load kinetics on both PL and WB were studied.

MATERIALS AND METHODS

Clinical specimens

Performance of CAP/CTM was retrospectively assessed on 185 residual PL specimens from 41 immunosuppressed individuals (11 solid organ transplant patients, including seven kidney, two liver and two lung transplant recipients; 14 hematopoietic stem cell transplantation patients; 7 HIV-infected subjects with full-blown AIDS and 9 onco-hematology patients) under surveillance for CMV infection whose WB samples were prospectively submitted for CMV viral load testing on WB to the Microbiology and Virology Unit, Azienda Ospedaliera Città della Salute e della Scienza di Torino and to the Microbiology and Virology Unit, Ospedale Amedeo di Savoia, Torino, Italy. Transplant recipients were monitored weekly for WB-CMV DNA during the first 3 months of follow-up, then every three months up to 1 year post-transplantation. In case of CMV DNA positivity, specimens were collected twice a week to monitor the rate of viral load increase and in patients undergoing pre-emptive treatment until two consecutive PCR negative determinations to monitor antiviral response. Pre-emptive antiviral therapy was initiated for CMV DNA levels ≥10,000 copies/mL or according to clinical evaluation. AIDS patients were routinely monitored for CMV infection and underwent specific anti-CMV treatment at the first CMV DNA positive specimen. In particular, from 2 to 14 samples for each patient were retrospectively tested according to the duration of the entire episode of CMV active infection and the availability of the specimens. Overall, 54 samples were collected from solid organ transplant patients, 78 samples from hematopoietic stem cell transplantation patients, 36 samples from onco-hematology patients and 17 specimens from AIDS patients. Prospectively collected blood samples in EDTA tubes were processed for nucleic acid extraction within 6 h. Left-over plasma was obtained by centrifugation at 2,000 x g for 15 min, then stored at -80°C until tested by CAP/CTM CMV (maximum storage duration of two months). Data of patients (*Table 1*), including pre-emptive treatment, were recorded in a specific database.

Table 1 - *Categories of patients enrolled in the study.*

Immunosuppressed patients TOT= 41	*No. (%) of patients*
Transplant recipients	25(61.0%)
Types of transplants	
SOT[1]	11(44%)
Liver	2(18.2%)
Kidney	7(63.6%)
Lung	2(18.2%)
HSCT[2]	14 (56%)
CMV pre-emptive treatment	
Yes	18(72%)
GCV[3]	7(38.9%)
VGCV[4]	4(22.2%)
FOS[5]	1(5.5%)
Sequential GCV[3]/VGCV[4]	1 (5.5%)
Sequential GCV[3]/FOS[5]	5(27.8%)
No	7(28%)
HIV/AIDS patients	7(17%)
Onco-hematology patients	9(22%)

[1]Solid organ transplantation
[2]Hematopoietic stem cell transplantation
[3]Ganciclovir
[4]Valganciclovir
[5]Foscarnet

CMV DNA QUANTIFICATION WITH REAL-TIME PCR

COBAS(®) AmpliPrep/COBAS(®) TaqMan(®) CMV test (CAP/CTM CMV test)

CAP/CTM CMV test was designed as a fully automated and integrated system from nucleic acid extraction to the final PCR result for high-throughput laboratories for the detection of CMV DNA in PL with the possibility of automatic transfer of the plates from the extractor to the amplifier (Roche Molecular Systems, Inc. COBAS TaqMan CMV Test FDA approved package insert. Branchburg, NJ: Roche, July 2012). Fully automated extraction from PL samples includes an initial lysis step, followed by nucleic acid fixation to magnetic beads, purification by a series of washing steps in which the beads are captured by a magnet, and immersion in hot elution buffer to release the nucleic acid. An internal quantitation standard (QS) is added to each sample to monitor the efficiency of the process. The COBAS AmpliPrep extractor automatically distributes the reaction mixture and DNA extract in a multiwell plate and transfers each specimen to the COBAS TaqMan for real-time PCR amplification. Amplification relies on a TaqMan real-time PCR and the target sequence is represented by CMV DNA polymerase (UL54) gene. PL-CMV DNA values are expressed as copies/mL. The limit of detection is 61 copies/mL with a dynamic range from 150 to 10x10E6 copies/mL and about 2 h of turnaround time for 24 plasma specimens.

QIAsymphony/ELITe Major Groove Binding-CMV test (QIA/MGB CMV test)

QIA/MGB CMV test is an automated open platform for sample extraction coupled to a real-time PCR-based method. WB samples are placed into 24-tube-capacity carrier racks and loaded into the instrument. The fully automated extraction is based on the same principle as the CAP/CTM CMV system, operating in an open mode. In brief, after viral lysis and magnetic beads-mediated DNA capture and purification, the extractor integrates automated PCR assay setup by distributing the reaction mixture and DNA extract in a multiwell plate. Unlike CAP/CTM, the plate transfer to the amplifier is manual. The CMV ELITe MGB™ kit uses specific MGB probes with different real-time amplification kinetics than TaqMan probes and amplifies the CMV MIEA (UL123) gene. The limit of detection is 367 copies/mL with a linear range from 367 to 28.2x10E6 copies/mL.

To assess assay performance, two CMV DNA proficiency panels (10 PL samples for CAP/CTM and 10 WB samples for QIA/MGB CMV test) consisting of CMV DNA lyophilized samples from the Quality Control for Molecular Diagnostics (QCMD, www.qcmd.org, 2012) were processed with the two PCR systems.

Statistical analysis

For statistical analysis GraphPad software (2015 GraphPad Software, Inc. La Jolla, USA) was applied. The correlation between the two systems was determined by linear regression analysis and mean differences in quantitation for averaged logs by the Bland-Altman plot. Only viral loads positive by both assays were represented on the Bland-Altman graphs. Differences were considered significant for p value <0.05.

RESULTS

Performance of CAP/CTM was assessed on 185 PL specimens from 41 patients who underwent prospective follow-up for CMV DNA detection on WB with the QIA/MGB CMV test. CMV DNA was detected in PL from 166 (89.7%) samples, while WB was positive in 181 (97.8%) specimens (92% concordance between PL and WB, corresponding to 4 and 166 concordantly negative and positive specimens, respectively).

Table 2 - *Results obtained with the two assays referred to the type of specimen analyzed.*

	Plasma / Positive	*Plasma /Negative*
Whole blood / Positive	166 (89.7%)	15 (8.1%)
Whole blood /Negative	0	4 (2.2%)

Fifteen WB specimens (8.1%) were positive while PL was negative corresponding to samples withdrawn during the late course of antiviral therapy; no specimen was WB negative/PL positive (*Table 2*). The correlation between CAP/CTM and QIA/MGB CMV was good (r: 0.9575, 95% confidence interval: 0.9435 to 0.9680, p<0.0001). For the 166 concordantly positive specimens, mean viral load (± standard deviation) was 2.88±0.73 $\log_{10}$ in PL and 3.76 ± 0.85 $\log_{10}$ copies/mL in WB. Bland-Altman analysis disclosed a bias between the two assays corresponding to a mean quantitation difference of 1.09±0.83 $\log_{10}$ copies/mL, with 97% of tested specimens being within ±2 SD of the averaged $\log_{10}$ results (*Figure 1*).

Eighteen transplant recipients (72%) underwent antiviral treatment. *Figure 2* reports the CMV-DNA kinetics in both PL and WB within the same patient, including the period of antiviral administration for two of the four patients described. CMV DNA showed a similar trend on the two biological matrices, with a PL viral load constantly 1 log lower than in WB. Under antiviral therapy, a significant CMV DNA decline was observed in both PL and WB starting from the first week of treatment, with a lower level of CMV DNA persisting for a longer period in WB than in PL (*Figure 2 panels C and D*).

CAP/CTM analytical performance against the CMV DNA proficiency panel from the QCMD showed a close correlation between observed and expected values (r=0.9838,

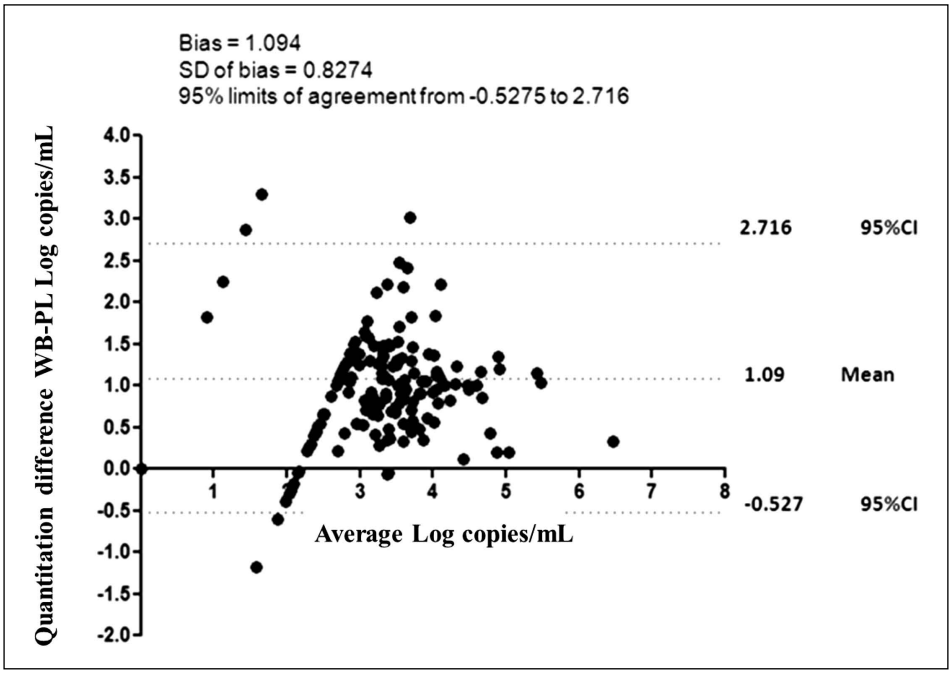

Figure 1 - *Bland-Altman analysis. Mean difference in quantitation obtained with the two assays: 1.09±0.83 log10 copies/ml.*

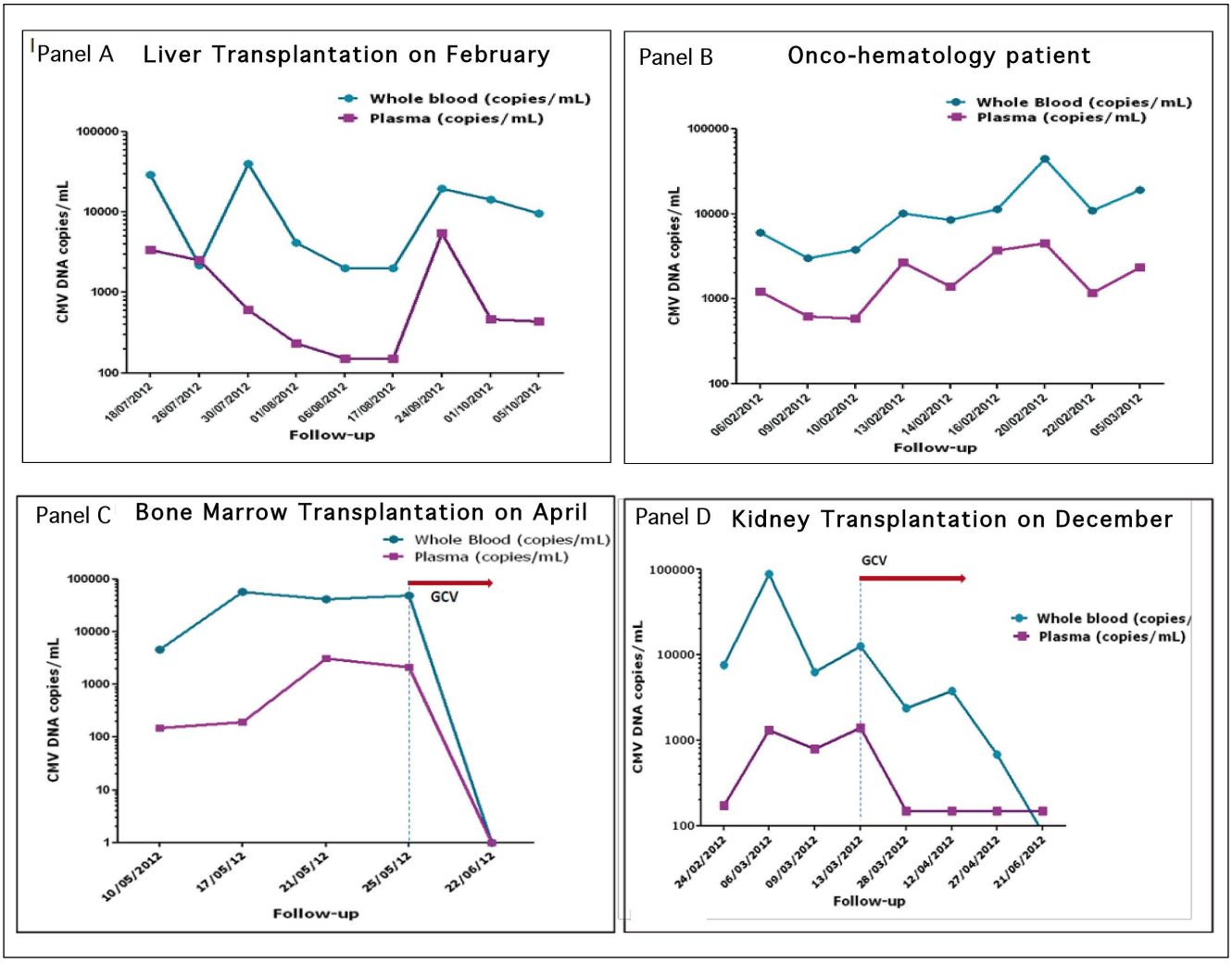

Figure 2 - *CMV DNA kinetics in plasma and whole blood from consecutive specimens within the same patient, including the period of antiviral administration for two of the four patients reported (GCV, ganciclovir; panels C and D).*

p<0.0001). QIA/MGB CMV performance on WB proficiency panel from QCMD was also very good (r=0.999, p<0.0001).

DISCUSSION

In immunosuppressed patients, CMV is responsible for life-threatening infections and pre-emptive anti-CMV therapy is widely used as the most effective strategy to control viral infection and reactivation. The success of pre-emptive anti-CMV therapy is related to CMV surveillance and viral load quantification with highly sensitive techniques for the early identification of viral replication (Boeckh and Boivin, 1998; Paya, 2001). Real-time PCR for CMV-DNA provides superior reproducibility and sensitivity than other non real-time PCR tests for the study of CMV dynamics during patient follow-up and allows antiviral treatment optimization. Established quantitative cut-offs predictive of active CMV disease, relapse risk and treatment duration are missing, due to the lack of a consensus on the biological matrix for CMV DNA detection and quantification (Caliendo *et al.,* 2009; Kraft *et al.,* 2012). CMV is a strictly cell-associated virus and WB is considered the most appropriate compartment for CMV-DNA quantification, reflecting both cell-associated and plasma-free virus. Quantification on WB is minimally affected by leucopenia, a major concern in critically ill patients. On the other hand, diagnostic procedures for testing viral load on WB are complex to standardize and prone to PCR inhibition due to the action of degraded hemoglobin products. However, this problem has now been limited using the available automated extraction and amplification procedures. Quantification of CMV-DNA in PL is simpler and faster, easy to perform and standardize, but limited by the lack of sensitivity compared to WB (Boivin *et al.,* 1998; Boivin *et al.,* 2000; Pang e *et al.,* 2003; Boeckh *et al.,* 2004; Piiparinen *et al.,* 2004; Loens *et al.,* 2007; von Muller *et al.,* 2007; Yerly *et al.,* 2007). Crucial for the standardization of quantitative PCR results on both PL and WB, particularly in high-throughput clinical laboratories is the fully integrated automation from DNA extraction to the final PCR results, now achieved by the majority of commercially available real-time PCR systems. In fact, nucleic acid extraction from biologic specimens is the most technically demanding step of PCR-based assays. In recent years, automated nucleic acid extraction systems applied to the detection of CMV-DNA improved the reliability and reproducibility of PCR results providing more accurate, reliable

and standardized results for clinical purposes (Mengelle *et al.,* 2003; Loens *et al.,* 2007; von Muller *et al.,* 2007; Costa *et al.,* 2014). A further step towards the standardization of quantitative CMV DNA results is the availability of the first international WHO standard and against it commercial PCR-assays should be all recalibrated (Hirsch *et al.,* 2013; Razonable *et al.,* 2013).

This study retrospectively studied the performances of the novel high through-put fully integrated commercially available real-time PCR platform CAP/CTM for CMV-DNA quantification on PL specimens from sequential samples of a series of immunosuppressed patients prospectively monitored on WB for CMV infection. PL results were compared to those obtained from WB in the same specimen from the same patient using a different PCR test (QIA/MGB CMV test) and CMV DNA kinetics on both compartments was studied. Data from the present study are consistent with a high performance of the fully integrated CAP/CTM CMV test both in clinical practice and from the analytical standpoint, thus confirming already published data on CAP/CTM CMV good performance (Kerschner *et al.,* 2011; Cardenoso *et al.,* 2013; Hirsch *et al.,* 2013; Pritt *et al.,* 2013; Mannonen *et al.,* 2014). The concordance between CAP/CTM and the QIA/MGB CMV test for CMV DNA quantification on both PL and WB was satisfactory. CMV DNA kinetics was similar on both biological matrices within the same patient, but PL viral load was constantly 1 log lower than in WB. These data support the suitability of both matrices for viral monitoring and the therapeutic management of CMV infection in immunosuppressed patients. However, quantitation differences were significant. Moreover, in patients under antiviral therapy, low levels of CMV DNA were detected more frequently in WB than in PL. This finding clearly shows the importance of the biological matrix in CMV DNA testing and how CMV DNA results depend on it.

In conclusion, CAP/CTM allows for an accurate and sensitive quantification of CMV DNA in PL. In high throughput laboratories the fully automated CAP/CTM system offers an added value due to a high level of standardization and integration of CMV DNA testing. Nevertheless, due to significant quantification differences between CMV viral load in PL and WB, the same biological specimen should be use for a sequential and reliable follow-up of patients at high risk of CMV infection.

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Abbreviations

CAP/CTM CMV test: COBAS(®) AmpliPrep/COBAS(®) TaqMan(®) CMV test

QIA/MGB CMV test: QIAsymphony/ELITe MGB-CMV test

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