Key features

Consistently high qPCR efficiencies (95% – 105%) for commonly used housekeeping genes, independent of amplicon length and GC content

High sensitivity and broad dynamic range

Fast and easy-to-use protocol

Real-time PCR (qPCR) is the preferred method for DNA and cDNA quantification because of its high sensitivity, reproducibility, and wide dynamic range.[1]

Despite the precision of qPCR, conclusions drawn from gene expression experiments are often misleading due to differences in amplification efficiency between the gene of interest and the housekeeping gene(s).

Introduction

The pitfalls in performing relative quantification with non-uniform amplification efficiencies were demonstrated by Pfaffl, et al.[2] For example, a difference in PCR efficiency (ΔE) of 10% between target gene and reference gene falsely calculated differences in expression ratio of 7.2% in the case of $E_{Target} < E_{Ref}$ and 1083% in the case of $E_{Target} > E_{Ref}$ after 25 performed cycles.[2] Differences in amplification efficiency can result from either sub-optimal primer design and/or difficult amplicon sequence (e.g., high GC content).

KAPA SYBR® FAST qPCR Kits contain the first DNA polymerase engineered specifically for SYBR Green I-based qPCR through a process of molecular evolution. The KAPA SYBR DNA Polymerase exhibits improved speed, processivity, and robustness resulting in consistently high amplification efficiencies required for accurate relative quantification. To demonstrate the high performance of the KAPA SYBR FAST qPCR Kit for gene expression analysis, the reaction efficiencies obtained for ten commonly used housekeeping genes in the human breast cancer cell line, MCF-7, were compared. The KAPA SYBR FAST qPCR Kit achieved consistently high amplification efficiencies (95% – 104%) across all ten genes, despite differences in amplicon length and GC content. The data below illustrates typical differences between the KAPA SYBR FAST qPCR Kit and a competitor kit (containing wild-type *Taq* DNA polymerase) with regards to efficiency, speed and sensitivity in the amplification of commonly used housekeeping genes. Data for two of the ten genes assessed in this study (ActB and HPRT1, with diverse GC contents) are shown in Figure 1 on the following page.

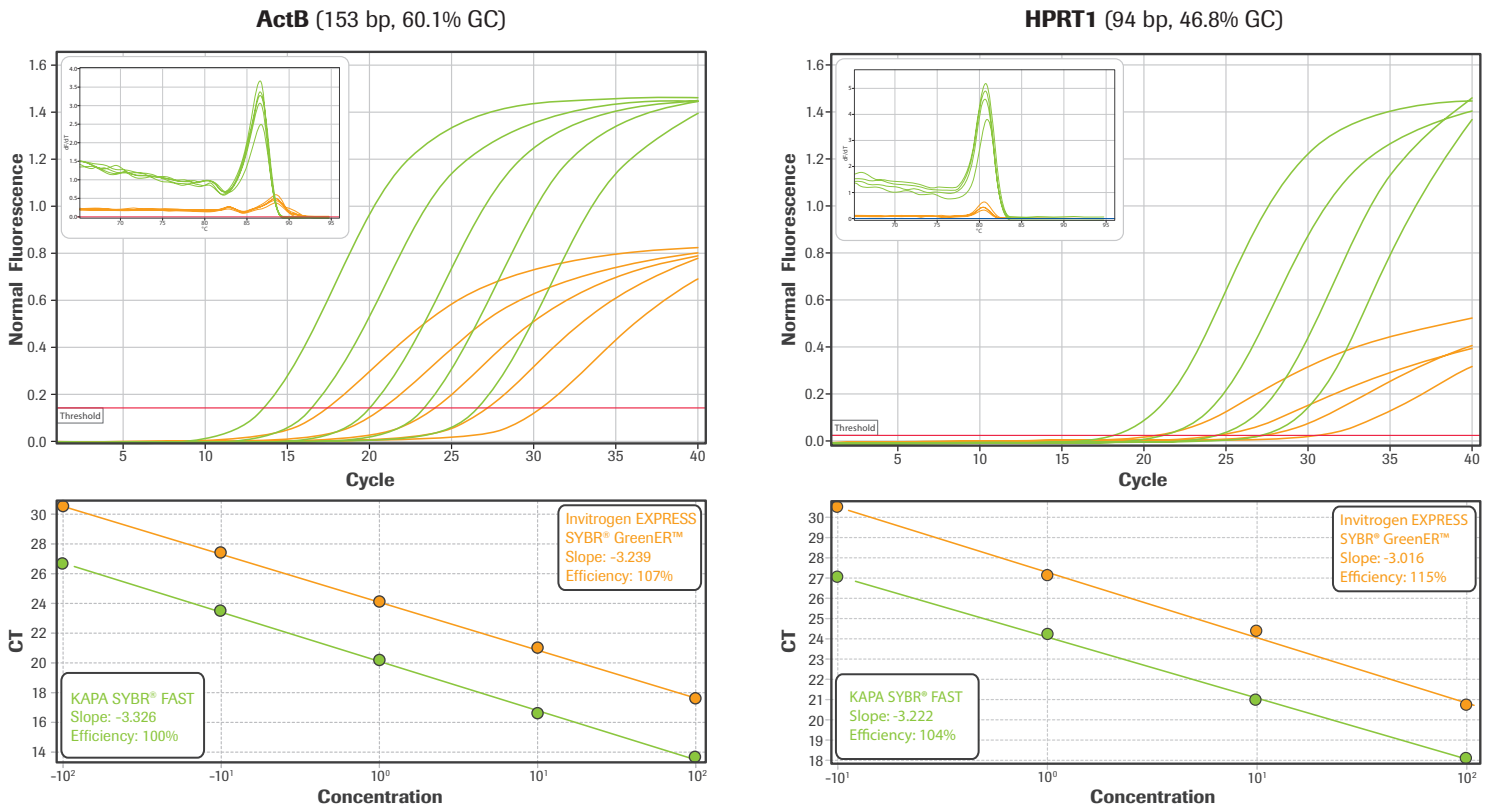

Figure 1. Typical results achieved with KAPA SYBR® FAST qPCR Kits in the amplification of housekeeping genes for gene expression analysis. Two housekeeping genes (ActB and HPRT1) were amplified from log-fold serial dilutions of MCF-7 cDNA (100 ng – 10 pg/reaction) using the KAPA SYBR FAST Universal qPCR Kit (green) or the Invitrogen Express SYBR® GreenER™ Kit (orange). Linear amplification plots (top) demonstrate earlier CT scores and greater baseline subtracted fluorescence for both the ActB and HPRT1 genes with the KAPA SYBR FAST Kit. Calculated reaction efficiencies (bottom) confirmed that the consistently high performance required for accurate expression quantitation is achievable with the KAPA SYBR FAST qPCR Kit (EActB=100% and EHPRT1=104%). Efficiencies obtained with the Invitrogen Express SYBR GreenER™ Kit were sub-optimal for both housekeeping genes (EActB=107% and EHPRT1=115%).

Experimental model and typical results

Ten commonly used housekeeping genes, representing a range of amplicon lengths and GC contents were selected for this gene expression analysis study. To reduce the effect of genomic amplification on expression results, primers were designed to target different exons of each gene or to span exon-intron boundaries. Amplicon and primer details may be found in Table 1.

Total RNA was isolated from the human breast cancer cell line, MCF-7, using the NucleoSpin® RNA II kit (Macherey-Nagel). RNA was digested with DNase I to remove contaminating genomic DNA. The ImProm-II™ Reverse Transcription System (Promega) was used to generate cDNA from 1 µg RNA with oligo (dT) primers. The cDNA was used as template to determine the amplification efficiency for each housekeeping gene using the KAPA SYBR FAST Universal qPCR Kit, according to **Protocol**.

The reaction efficiency achieved for each housekeeping gene was calculated using the CT slope method, with five data points corresponding to log-fold MCF-7 cDNA serial dilutions (100 ng – 10 pg/reaction). Results (average of triplicate determinations) for all ten housekeeping genes are summarized in Table 1. Consistent, high amplification efficiencies (95% – 104%) were achieved in all cases. Typical results (linear amplification, dissociation, and standard curves) of four of the ten housekeeping genes (YWAZ, RPL13a, GAPDH, and HMBS) are given on the next page.

Table 1: Primer sequences and assay results for the ten housekeeping genes analyzed in this study

Amplicon	Locus	GC (%)	Amplicon length (bp)	Slope	Efficiency (%)	Primer sequences
SDHA[3]	5p15	44.2	86	-3.316	100	F: 5'- TGG GAA CAA GAG GGC ATC TG -3' R: 5'- CCA CCA CTG CAT CAA ATT CAT G -3'
YWHAZ	2q25	44.6	249	-3.429	96	F: 5'- GCA ACC AAC ACA TCC TAT CAG AC -3' R: 5'- CCT CCT TCT CCT GCT TCA GC -3'
B2M	15q21-q22.2	45.3	139	-3.416	96	F: 5'- GTA TGC CTG CCG TGT GAA C -3' R: 5'- AAA GCA AGC AAG CAG AAT TTG G -3'
HPRT1[3]	Xq26.1	46.8	94	-3.222	104	F: 5'- TGA CAC TGG CAA AAC AAT GCA -3' R: 5'- GGT CCT TTT CAC CAG CAA GCT -3'
TBP	6q27	50.3	137	-3.355	99	F: 5'- TGC CCG AAA CGC CGA ATA TAA TC -3' R: 5'- GTC TGG ACT GTT CTT CAC TCT TGG -3'
UBC	12q24.3	52.0	123	-3.361	98	F: 5'- CGG GAT TTG GGT CGC AGT TCT TG -3' R: 5'- CGA TGG TGT CAC TGG GCT CAA C -3'
RPL13a	19q13.3	52.8	231	-3.459	95	F: 5'- AAG GTC GTG CGT CTG AAG -3' R: 5'- GAG TCC GTG GGT CTT GAG -3'
GAPDH	12q13	55.0	138	-3.392	97	F: 5'- CAG GAG GCA TTG CTG ATG AT -3' R: 5'- GAA GGC TGG GGC TCA TTT -3'
ActB	7q15-p12	60.1	153	-3.328	100	F: 5'- CGG CAT CGT CAC CAA CTG -3' R: 5'- AAC ATG ATC TGG GTC ATC TTC TC -3'
HMBS	11q23.3	62.5	200	-3.320	100	F: 5'- AGG AGT TCA GTG CCA TCA TC -3' R: 5'- GCA GCG AAG CAG AGT CTC -3'

As indicated in Figure 2, no bias in amplification efficiency relative to GC content (44.2% – 62.5%) or amplicon length (86 – 249 bp) was observed. Collectively, this data confirms that KAPA SYBR® FAST qPCR Kits are ideally suited for gene expression quantitation.

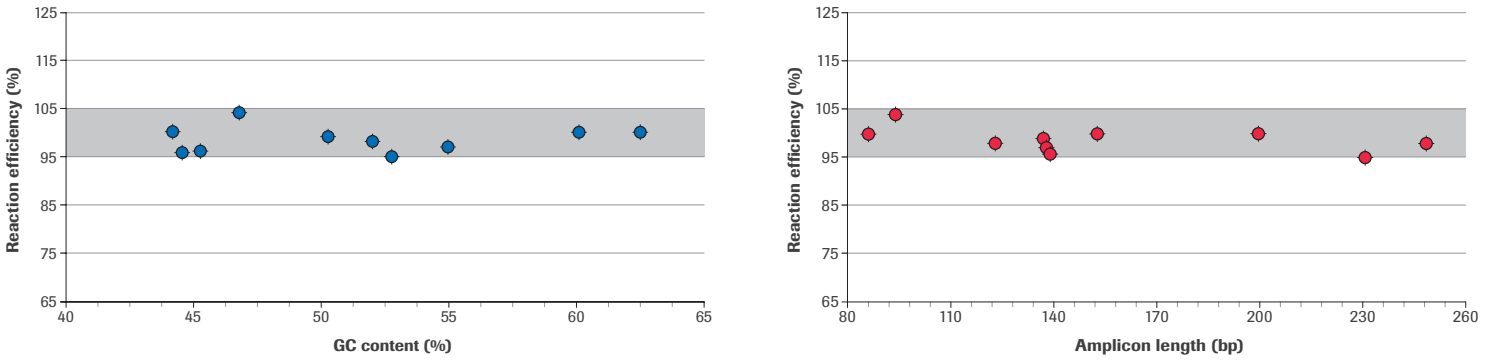

Figure 2. Amplification efficiencies achieved for ten housekeeping genes with the KAPA SYBR FAST Universal qPCR Kit, plotted against GC content (left) or amplicon length (right). The reaction efficiency achieved for each of the ten genes fell within the optimal range of 95 – 105%, independent of the nature or length of the amplicon.

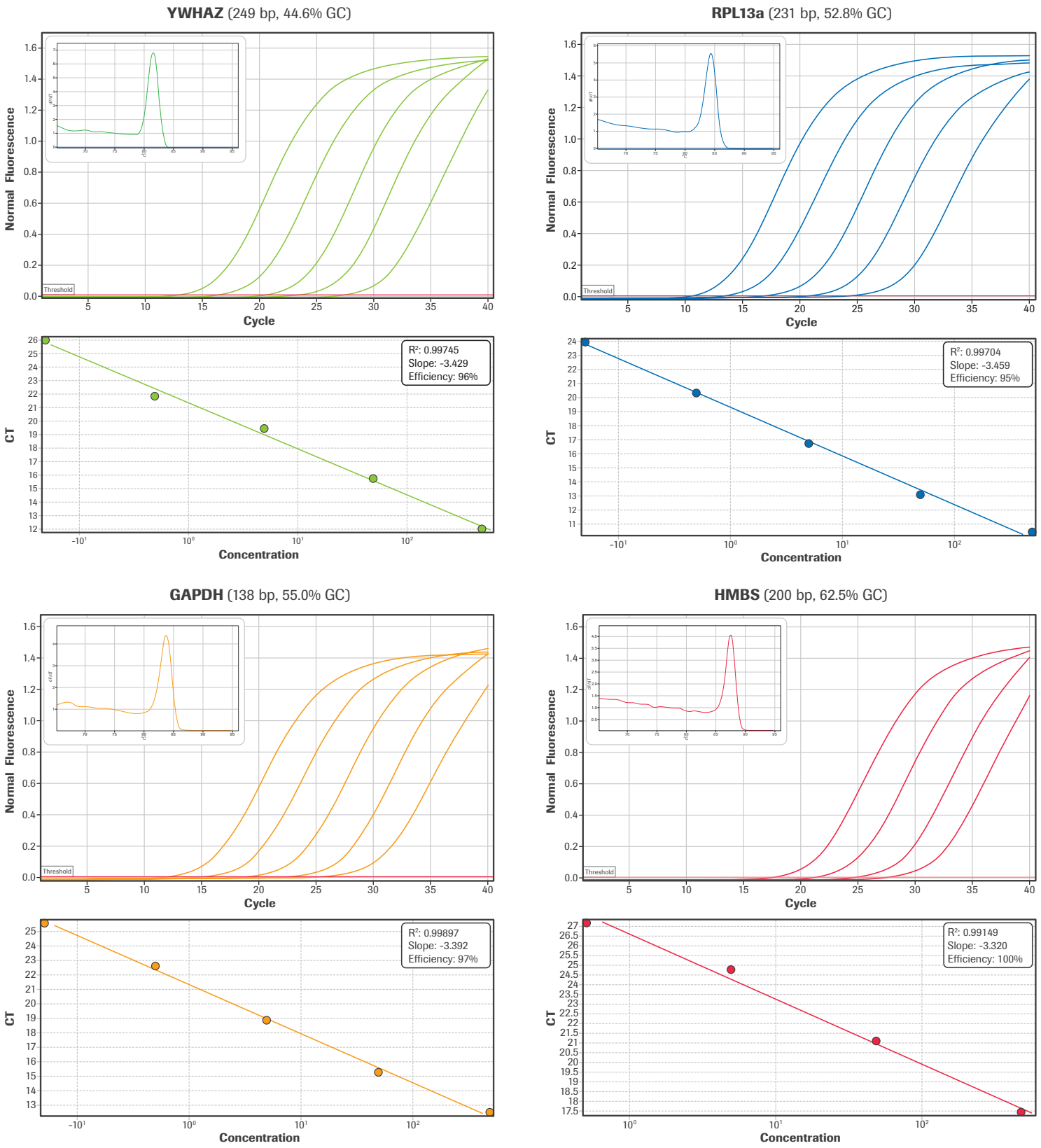

Figure 3. The data shown below (for housekeeping genes YWHAZ, RPL13a, GAPDH, and HMBS) represent a range of amplicon lengths (138 – 249 bp) and GC contents (44.6% – 62.5%). Genes were amplified from five log-fold serial dilutions of MCF-7 cDNA (100 ng – 10 pg/20 µL reaction), as outlined in Sections 2 and 3. Linear amplification plots for each of the housekeeping genes demonstrate accurate amplification of serially diluted cDNA. Dissociation plots of the last amplifiable dilution (insets) indicate specific amplification of each gene. Reaction efficiencies, determined using the CT slope method, demonstrate consistently high, unbiased amplification across a diverse range of amplicons (EYWHAZ=96%, ERPL13a=95%, EGAPDH=97%, and EHMBS=100%), which is essential for meaningful gene expression analysis. For additional data relating to these examples and the other six housekeeping genes included in this study, please refer to Table 1 on the previous page.

Protocol

1. RNA extraction

- RNA may be extracted from a suitable source with any commercially available RNA extraction kit.
- Always treat extracted RNA with RNase-free DNase I prior to cDNA synthesis to eliminate contamination with genomic DNA.
- Determine the quantity and quality of RNA prior to cDNA synthesis using a UV spectrophotometer, Agilent BioAnalyzer or Bio-Rad Experion. Do not use RNA of a low quality for cDNA synthesis.

2. cDNA preparation

- Use 1 – 5 µg total RNA or mRNA free of contaminating DNA per reaction.
- cDNA may be prepared with any commercial cDNA synthesis kit.
- Determine the quantity and quality of cDNA prior to qPCR using a UV spectrophotometer, Agilent BioAnalyzer or Bio-Rad Experion. Do not use low-quality cDNA for gene expression analysis.
- Aliquot cDNA for long-term storage at -20°C or -80°C. For optimal results, avoid repeated freeze/thaw cycles.

3. qPCR reaction setup and cycling protocol

- Select the appropriate KAPA SYBR® qPCR Kit for the instrument in use (Universal, ABI Prism®, Bio-Rad iCycler™, or Roche LightCycler® 480). For more details on reaction setup and cycling parameters, please refer to the Technical Data Sheet for each specific kit.
- Prepare five log-fold dilutions of cDNA (starting at 100 ng/µL). This is sufficient for efficiency curve analysis for each primer pair using the CT slope method. Include a No Template Control (NTC) in each template dilution series.
- For optimal results, perform cDNA dilutions in 10 mM Tris-HCl +0.1% Tween 20®. Accurate pipetting is essential.
- The recommended volumes/final concentrations of reaction components are given in the following table. For optimal results, prepare a reaction master mix containing all components, except template, for each primer pair. Prepare a sufficient volume of each reaction master mix to assay every cDNA dilution in triplicate for each primer pair.
- Aliquot 19 µL of reaction master mix into the wells of a PCR plate or PCR tubes, followed by 1 µL of the appropriate cDNA dilution. Cap or seal plates/tubes and mix or centrifuge briefly.
- Perform the qPCR using the appropriate cycling protocol. The cycling parameters given below are optimized for the specific primer pairs used in this study, using the Corbett RotorGene 6000 HRM qPCR instrument.

Reaction component	Final conc.	Per 20 µL rxn
PCR grade water	–	7.8 µL
2x KAPA SYBR FAST qPCR Master Mix	1x	10 µL
Forward primer (10 µM)	0.3 µM	0.6 µL
Reverse primer (10 µM)	0.3 µM	0.6 µL
Template cDNA (100 ng – 10 pg/rxn)		1 µL
Total volume:		**20 µL**

Cycling step	Temperature and time	
Initial denaturation	3 min at 95°C	
Denaturation	10 sec at 95°C	40 cycles
Annealing	30 sec at 55°C	
Extension	1 sec at 72°C Data acquisition point	
Melt	Ramp from 65°C to 95°C	

4. Data analysis

- Confirm that all DNA dilutions used for reaction efficiency calculations yield a specific product with a single expected melting profile. Confirm that specific product with a melting profile identical to that of the target cDNA amplicon was not amplified in NTC reactions.
- Calculate reaction efficiency using the CT slope method (use instrument guidelines). If the reaction efficiency is <95% or >105%, re-optimize cycling conditions.
- Use the 100 ng cDNA/reaction samples for relative quantification analysis.

References and acknowledgements

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2. Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002). *Nucl. Acids Res.* 30(9): e36.
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