Video Article

Rapid Verification of Terminators Using the pGR-Blue Plasmid and Golden Gate Assembly

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Abstract

The goal of this protocol is to allow for the rapid verification of bioinformatically identified terminators. Further, the plasmid (pGR-Blue) is designed specifically for this protocol and allows for the quantification of terminator efficiency. As a proof of concept, six terminators were bioinformatically identified in the mycobacteriophage Bernal13. Once identified, terminators were then made as oligonucleotides with the appropriate sticky ends and annealed together. Using Golden Gate Assembly (GGA), terminators were then cloned into pGR-Blue. Under visible light, false positive colonies appear blue and positively transformed colonies are white/yellow. After induction of an arabinose inducible promoter (pBad) with arabinose, colony strength can be determined by measuring the ratio of green fluorescent protein (GFP) produced to red fluorescent protein (RFP) produced. With pGR-Blue, the protocol can be completed in as little as three days and is ideal in an educational setting. Additionally, results show that this protocol is useful as a means for understanding *in silico* predictions of terminator efficiency related to the regulation of transcription.

Video Link

The video component of this article can be found at http://www.jove.com/video/54064/

Introduction

Large synthetic biology projects necessitate the use of highly effective transcription terminators to help regulate gene expression. Identification of novel terminators requires bioinformatic analysis of novel genomes. However, as increasing amounts of bioinformatic software are developed, each with a unique algorithm utilized for prediction, more discrepancy between putative results occurs. Because this process is somewhat subjective and is done *in silico*, these predictions need biological confirmation.[1] Additionally, the volume of putative terminators identified through *in-silico* analysis requires the use of cloning strategies that can be completed in a relatively short time frame.

The PGR-Blue plasmid is a modification of the PGR plasmid that has been redesigned to use Golden Gate Assembly (GGA) to simplify the cloning procedure by allowing for all reaction steps to be simultaneously performed in one micro-centrifuge tube.[2,3] Color selection was incorporated into the plasmid to increase the ease of identifying positive colonies. A successful ligation should be white/yellow in visible light and fluoresce green under blue (450 nm) or ultraviolet (UV) light when grown on plates containing arabinose. Because uncut pGR-blue contains a blue chromo protein (amilCP), colonies containing an unmodified plasmid are blue under visible light. This simplification along with the streamlined protocol allows researchers to proceed from bioinformatic identification to biological confirmation in three to four days. The design nature of this system can be beneficial both in the research lab and in educational settings.

The pGR-Blue plasmid allows for quantification of terminator strength.[4] A single arabinose inducible promoter is used to produce green fluorescent protein (GFP) and red fluorescent protein (RFP). The terminator is cloned into the plasmid after the GFP sequence but before the RFP sequence, thus stopping the transcription of the RFP protein. The strength of the terminator is determined by the ratio of GFP produced to RFP produced.

The Vision and Change[5] report suggested that Science, Technology, Engineering and Math (STEM) education incorporate research based experiences into the classroom.[6] However, this requires the development of protocols that can be done by students with limited skill sets in a defined time frame. While the protocol can be accomplished in as little as three days, it was also designed so that each major step could be accomplished in a separate weekly (2-3 hr) lab period to create a Course Research Experience (CRE). When used in this manner, the procedure will take between three and six weeks and is appropriate for both introductory and advanced courses in Genetics, Cell Biology or Bioinformatics.

Protocol

1. Designing and Ordering Oligonucleotides with the Appropriate Sticky Ends

1. Identify potential rho-independent terminators through genomic analysis using programs that are freely available online.[7]
2. When working with double stranded DNA, determine the orientation of the terminator to be tested.[7] The pGR-Blue plasmid only verifies terminators ligated in the 5' to 3' direction on the top (forward strand).
    1. Convert a bottom (reverse) strand terminator to its reversed complement to reorient the sequence for testing in pGR-Blue using free online software, *e.g.*, ApE.
3. After the orientation is determined to be correct, add the sticky end " 5'-CGAC-3' " to the 5' end of the top strand. Add the sticky end " 5'-CCGC-3' " to the 5' end of the bottom strand. [8] This will ensure that ligated insert will be inserted in the appropriate orientation using GGA.
4. Once sequences are determined, order as single stranded oligonucleotides commercially.

2. Annealing Oligonucleotides (Applies Only to Freeze-dried DNA)

1. Re-suspend individual oligonucleotides in nuclease free water to a concentration of 100 µM.
2. Make 10x annealing buffer: 1 M NaCl and 100 mM Tris-HCl pH 7.4. Annealing buffer can be stored at 4 °C for several weeks. Once annealing buffer is made and oligonucleotides are re-suspended, begin the annealing[8].
3. For each terminator to be tested, add 16 µl of ultra-pure water to a 1.5 ml microcentrifuge tube.
4. Add 2 µl of 10x annealing buffer to each microcentrifuge tube.
5. Add 1 µl of the top strand oligonucleotide (100 µM) from the desired terminator.
6. Add 1 µl of the bottom strand oligonucleotide (100 µM) from the desired terminator.
7. Create a boiling water bath using a 1,000 ml beaker containing approximately 400 ml of tap water and place on a hot plate.
8. Place the microcentrifuge tubes from step 2.6 in a float and boil for 4 min. After 4 min, turn off the heat plate but leave the tube and float in the water bath to slowly cool O/N (18 hr) [8]. Store the annealed terminators at -20 °C.

3. Golden Gate Assembly (Standard Mix — Cost Efficient)

1. Make a 40 nM dilution of the annealed terminators. Add 124 µl of nuclease free water to 1 µl of annealed terminators mix (100 µM).
    1. Centrifuge samples for 30 sec at 10,000 x g and store on ice.
2. Into a new tube appropriate for the thermocycler being used, add 6 µl of nuclease free water.
3. Add 1 µl of commercial DNA Ligase Buffer [300 mM Tris-HCl (pH 7.7-7.8), 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP]. Perform all subsequent steps on ice.
4. Add 1 µl of pGR-Blue (35-50 ng/µl) destination plasmid to the microcentrifuge tube.
5. Add 1 µl of the 40 nM annealed terminators to the microcentrifuge tube.
6. Add 0.5 µl commercial high-fidelity BsaI restriction endonuclease to the microcentrifuge tube.
7. Add 0.5 µl of commercial DNA ligase to the microcentrifuge tube.
8. Centrifuge the reaction mixture at 10,000 x g for 120 sec. Then place the tube directly into the thermocycler.
9. Run the thermocycler program: 20 cycles of 1 min at 37 °C followed by 1 min at 16 °C, followed by 1 cycle of 15 min at 37 °C. Use samples immediately or freeze and store at -20 °C.[8]

4. Golden Gate Assembly (Commercial Master Mix — Time Efficient)

1. Add 124 µl of nuclease free water to 1 µl of the 100 µM annealed terminators created in step 2. This dilutes the terminator DNA concentration to 40 nM. Perform this step in a new, labeled 1.5 ml microcentrifuge tube.
2. Into a new tube appropriate for the thermocycler, add 14 µl of nuclease free water. Make sure the new tube is labeled accordingly.
3. Add 2 µl of Commercial Master Mix Buffer to the microcentrifuge tube.
4. Add 1 µl of pGR-Blue plasmid (35-50 ng/µl) to the microcentrifuge tube.
5. Add 2 µl of the 40 nM annealed terminators to the microcentrifuge tube.
6. Add 1 µl of Commercial Master Mix to the microcentrifuge tube.
7. Centrifuge the 40 nM tube at 10,000 x g for 120 sec. Then place the tube directly into the thermocycler.
8. Run the thermocycler program: 1 cycle of 60 min at 37 °C followed by 1 cycle of 15 min at 55 °C. Use samples immediately or freeze and store at -20 °C.

5. Transformation and Colony Selection

1. Prepare Luria Base (LB) agar Petri plates containing 1 mM concentration of ampicillin (Amp) and a 10 mM concentration of arabinose (arb). Prepare at least 10 ml of 1 M *L*-arabinose stock solution because this stock solution will be used in later steps.
2. Use any high-efficient (10^8 - 10^9 transformants/µg) chemically competent *E. coli* (JM109) cells for transformation.[9] For best results, add 3-5 µl of the ligation reaction to 50 µl of competent cells. When using chemical competent cells, follow the transformation protocol specific to the cells being used.
3. Spread half (25 µl) of the transformed cells onto pre-warmed LB (Amp/arb) agar plates using a sterile L-shaped "hockey stick" and incubate O/N at 37 °C. Due to ampicillin's rapid rate of decomposition, do not incubate at 37 °C over 24 hr.

4. Perform colony selection based on color by visual inspection. A successful ligation should be white/yellow in visible light and fluoresce green under blue (450 nm) or UV light. An unsuccessful ligation will produce a colony that is blue in color under visible light after 18 - 20 hr.

6. Verification and Quantification of Terminator

1. Prepare 5 ml sterile LB-broth tubes with 1 mM ampicillin and 10 mM arabinose. The number of tubes needed is dependent on the number of terminators being tested plus controls (cells with ampicillin resistance that do not fluoresce and cells containing the uncut pGR-Blue plasmid).
2. Under visible light pick 2-3 white/yellow colonies (step 5.4) using a sterile loop. Allow the samples to incubate in broths on a shaker at 37 °C and 160 rpm for 16-18 hr. Do not incubate these samples for more than 24 hr.
   1. Optionally, use a spectrophotometer to determine optical cell density at 600 nm (OD_{600}) to ensure adequate cell growth. Generally, an OD_{600} of ~0.8-1.0 is observed after 16-18 hr of growth.
3. Use a sterile 96-well plate for the remaining steps. Conduct these steps in an aseptic environment and in triplicate. Add 199 µl of sterile LB+ Amp/arabinose broth (1 mM ampicillin/10 mM arabinose) to each well. Include room on the plate for three separate controls.
   1. Use the following three controls: A well (1) containing LB + Amp/arabinose broth only and a well (2) for untransformed competent cells (blank) to serve as a blank. For quantification (3) of the terminator strength, cells containing uncut original pGR or containing pGR-Blue ligated with a non-terminating sequence should be included (reference control for quantification). See **Figure 1** for plate reader layout.
4. Pipet 1 µl of the cell solution from the O/N broths into individual wells of the 96-well plate.
5. Seal plates with a breathable cover and shake for 18-20 hr at 37 °C and 160 rpm.
6. Using a plate reader, determine OD_{600} by reading absorbance at 600 nm. Measure GFP fluorescence as follows: excitation 395 and emission 509. Measure RFP fluorescence as follows: excitation 575 and emission 605. After 18-20 hr, the OD_{600} should be approximately 0.5.
7. To normalize for variation in growth, use the equation: $\frac{Fluorescence}{OD\,600}$ for both GFP and RFP.
8. After normalization, determine relative terminator strength using the equation $\frac{GFP_{terminator}}{GFP_{control}} \div \frac{RFP_{terminator}}{RFP_{control}}$.

Representative Results

This protocol will produce cells containing pGR-Blue with a terminator ligated between GFP and RFP using Golden Gate Assembly (**Figure 2**). Positive colonies containing ligated insert can be selected based on color. In visible light positive colonies will be white/yellow and false positives will produce blue colonies after 18-20 hr of incubation at 37 °C (**Figure 3**).

After colony selection, a plate reader can be used to determine terminator strength. As proof of concept, six putative terminators (T1-6) were bioinformatically identified in the mycobacteriophage Bernal13. The results in **Figure 4** show relative terminator strength with cells expressing uncut/unmodified pGR as a control. If a predicted terminator has a GFP/RFP ratio similar to pGR (~0.0) or below 0.1, then the sequence was determined to not be a terminator (T5 and T6). However, numerically each tenth increase represents a fold increase in terminator strength relative to the control. For example, T3 had a high ratio of GFP expression relative to RFP and showed a 5-fold increase (0.5 on graph) in terminator strength as compared to the control and was considered a strong terminator (**Figure 4**). Data was collected in triplicate and averaged for visual representation.

To determine the best reference control for quantitating terminator strength, we compared raw data using the original pGR plasmid containing no terminator and pGR-Blue cloned with a sequence known not be a terminator. Both controls showed similar levels of GFP and RFP expression (**Figure 5**) and, more importantly, produced similar GFP/RFP ratios.

| Contamination Control	Cell Growth Control / Blank	GFP/RFP normalization control			
(1) LB-broth Only	(2) Untransformed Cells	(3) Cell transformed with pGR (only)	Terminator A	Terminator B	Terminator C
(1) LB-broth Only	(2) Untransformed Cells	(3) Cell transformed with pGR (only)	Terminator A	Terminator B	Terminator C
(1) LB-broth Only	(2) Untransformed Cells	(3) Cell transformed with pGR (only)	Terminator A	Terminator B	Terminator C

Figure 1. Schematic Detailing Plate Reader Layout. Room should be included on the plate reader for three separate controls (Green). A well (1) containing LB + Amp/arabinose broth only and a well (2) for untransformed competent cells (blank) to serve as a blank. For quantification (3) of the terminator strength, cells containing uncut original pGR or containing pGR-Blue ligated with a non-terminating sequence should be included (reference control for quantification). The remainder of the plate (yellow) can be used to test putative terminators. It is suggested all cells be grown in triplicate and averaged before analysis. Please click here to view a larger version of this figure.
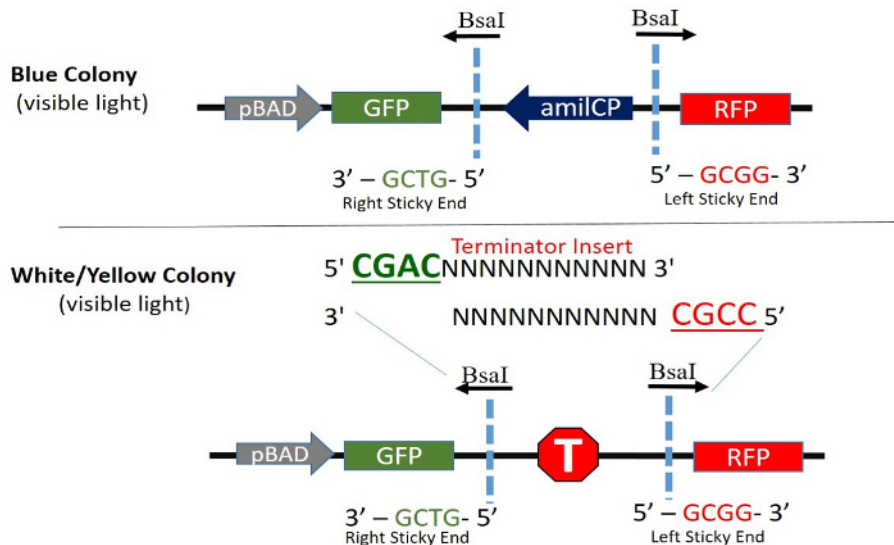
Figure 2. Cloning Site Orientation and Expression of pGR-Blue. Constitutive expression of AmilCP (Blue Chromo Protein) is driven in the opposite (bottom strand) direction of GFP and RFP. When digested with BsaI, AmilCP and the BsaI recognition sites are removed and the terminator to be tested is permanently ligated into the plasmid. The top strand and bottom strand of a terminator with the appropriate sticky ends are shown. The AraC regulator and ampicillin resistance genes are in the plasmid but not shown. Please click here to view a larger version of this figure.
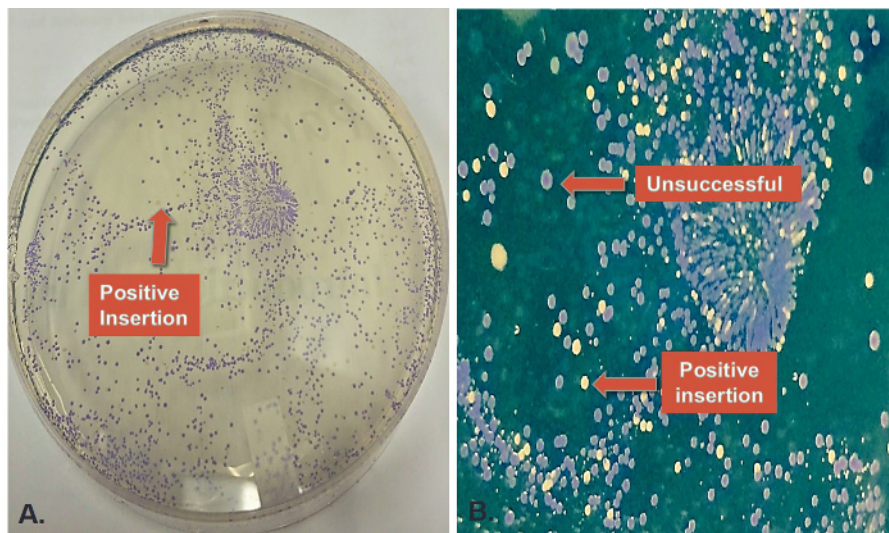
Figure 3. Colony Selection after Cloning. (A) Representative plate after pGR-Blue / terminator ligation (standard mix) and cell transformation. Blue colonies represent unsuccessful ligations and white/yellow colonies (indicated by arrow) are successful ligations. **(B)** A zoomed in image of the same plate showing representative colonies after transformation. The background color was digitally altered to further highlight colony color differences. Transformed cells (*E.coli*-JM109) were plated on LB agar plats containing ampicillin and arabinose. Please click here to view a larger version of this figure.
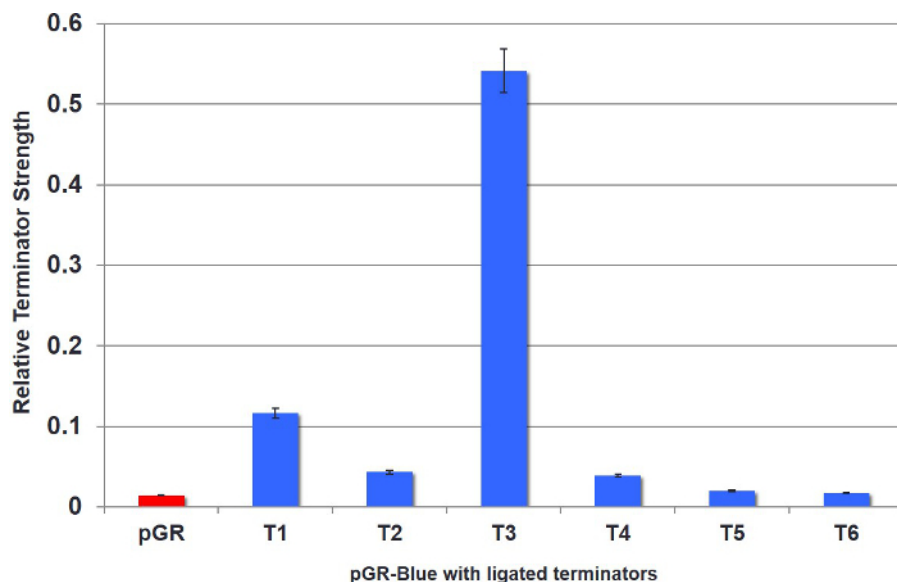
Figure 4. Quantification of Six Different Terminators in pGR-Blue. Six putative terminators (T1-6) were bioinformatically identified in the mycobacteriophage Bernal13. Results show relative fluorescence. All results were normalized using cells containing uncut/unmodified pGR as a control. T3 was found to be a strong terminator. All results represent individual colonies grown in triplicate and averaged for visual representation. Error bars represent deviations between averaged data points. Please click here to view a larger version of this figure.
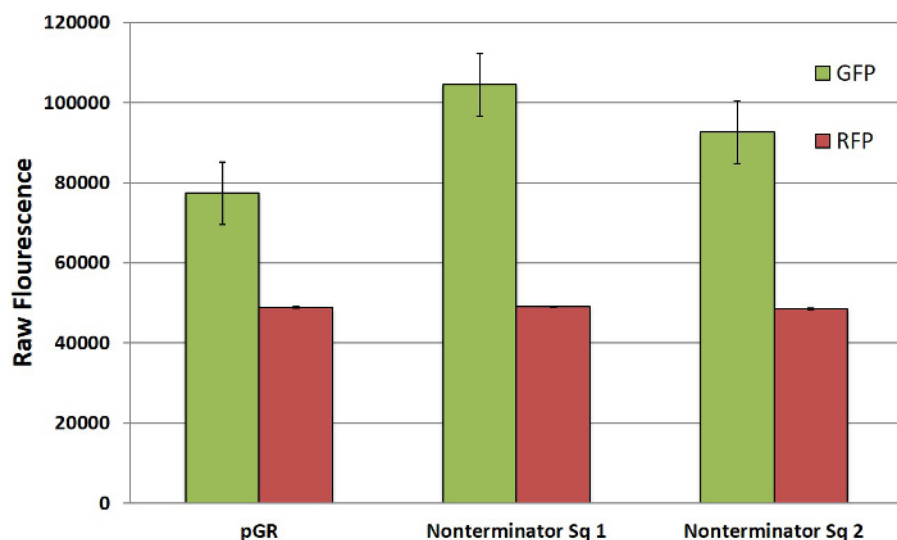
Figure 5. GFP and RFP Expression in Controls. Expression of GFP and RFP in uncut/unmodified pGR and two different non-terminator sequences inserted into pGR-Blue. All results represent individual colonies grown in triplicate and averaged for visual representation. Error bars represent deviations between averaged data points. Please click here to view a larger version of this figure.

Discussion

The most important step in this protocol is proper oligonucleotide design prior to ordering. The oligonucleotides must have the appropriate sticky ends added to the 5' ends of both the top and bottom strands to ensure that GGA incorporation is possible. Additionally, it is important to switch the orientation of left facing terminators (terminators that stop transcription on the bottom strand) to that of right facing (terminates transcription on the top strand) terminators because GFP and RFP expression is in the right facing (top strand) direction.

Two separate GGA protocols and buffer mixes can be used to test terminators in pGR-Blue. The standard-economy mix consists of material purchase individually and then mixed in the lab [8]. The second protocol uses a commercial pre-made GGA master mix. While both work well; the commercial master mix is more time-efficient but is relatively expensive compared to the standard mix. In an education setting, it may be more economical to make the standard GGA mix in advance and freeze aliquots for individual student use.

As a result of modifying pGR for GGA and adding amilCP in the reverse orientation for color selection, the expression of GFP and RFP is limited in the uncut pGR-Blue plasmid and should not be used as the reference control when determining relative terminator strength. However, all modifications are removed during the cloning process allowing for proper quantification of relative terminator strength. While it is best to use

the original unmodified pGR plasmid similar results can be achieved by cloning a sequence known not to be a terminator into pGR-Blue. Both controls showed equal levels of GFP and RFP expression (**Figure 5**) and, more importantly, had similar GFP/RFP ratios.

For tight regulation of gene expression, the pGR-Blue plasmid constitutively expresses the AraC regulator of the pBAD (arabinose inducible) promoter.[10,11] Depending on the cell line used a high arabinose concentration may be required for the expression of GFP and RFP. Good results were achieved using a concentration of 10 mM arabinose in the *E. coli* cell line JM109. Transformed cells should be either white/yellow or blue under visible light. Under UV light or blue light (450 nm), it is common to only see green fluorescence but not red in positively transformed cells. However, RFP fluorescence was detected using the plate reader.

The terminators used as proof of concept for this protocol were identified in the mycobacteriophage Bernal13. However, pGR-Blue only works in *E. coli* and it is not a shuttle vector that can be expressed in other species of bacteria. The result is that we do not know exactly how the identified terminators would function in their host environment and is a potential limitation. The expression cassette associated with pGR-Blue is flanked by traditional cloning sites and could be moved to an appropriate shuttle vector. This would allow individual researchers to tailor pGR-Blue to their specific bacterial species, if so desired.

While there are other high-throughput sequence analysis procedures available, most use a forward genetics approach. These approaches use site directed mutagenesis or oligo-synthesis to generate hundreds to thousands of potential sequence combinations.[12,13] Once created, the effect of each sequence modification is then cataloged. However, pGR-Blue is designed for a more directed reverse genetics application. When analyzing a novel genome *in silico*, it is common to observe multiple potential terminators in a given area. pGR-Blue was designed to help researchers quickly refine their *in silico* predictions by allowing them to test putative sequences *in vivo*. It should be noted the pGR terminator testing plasmid was used to quantitate terminator strength in 582 sequences[4], however pGR uses an restriction-enzyme assembly standard (*e.g.*, basic bio-brick assembly (BBA)), a relatively time consuming cloning process compared to GGA.[7] The pGR-Blue plasmid was designed to function similarly to pGR but uses a more rapid cloning procedure and colony color selection. These modifications, along with our simplified protocol, make pGR-Blue adaptable to both education and research oriented labs.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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