Chapter 15

Generating Mouse Models Using Zygote Electroporation of Nucleases (ZEN) Technology with High Efficiency and Throughput

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Abstract

Mouse models with genetic modifications are widely used in biology and biomedical research. Although the application of CRISPR-Cas9 system greatly accelerated the process of generating genetically modified mice, the delivery method depending on manual injection of the components into the embryos remains a bottleneck, as it is laborious, low throughput, and technically demanding. To overcome this limitation, we invented and optimized the ZEN (Zygote electroporation of nucleases) technology to deliver CRISPR-Cas9 reagents via electroporation. Using ZEN, we were able to generate genetically modified mouse models with high efficiency and throughput. Here, we describe the protocol in great detail.

Key words Mouse model generation, CRISPR-Cas9, Electroporation, Mouse zygotes, ZEN

1 Introduction

The type II CRISPR-Cas9 system of *Streptococcus pyogenes* works efficiently in generating mouse models with various genetic modifications. Specifically, the system can introduce targeted mutations during embryogenesis [1–5]. This ability to introduce targeted modifications in embryos can have a dramatic impact on studies investigating events in early development such as zygotic genome activation because genetically modified embryos can be generated without breeding. Although this new technology is highly versatile and efficient, the delivery of CRISPR-Cas9 components is still mainly dependent on the microinjection, which is technically demanding and labor intensive.

To overcome this limitation, we developed and optimized the Zygote Electroporation of Nuclease (ZEN) method [6, 7]. We demonstrated that the CRISPR-Cas9 components, including Cas9 mRNA or Cas9 protein, single guide RNA (sgRNA), and a DNA oligo donor, could be efficiently delivered into mouse zygotes by electroporation to generate mice with targeted genetic modifications (Fig. 1).

Kiho Lee (ed.), *Zygotic Genome Activation: Methods and Protocols*, Methods in Molecular Biology, vol. 1605,
DOI 10.1007/978-1-4939-6988-3_15,

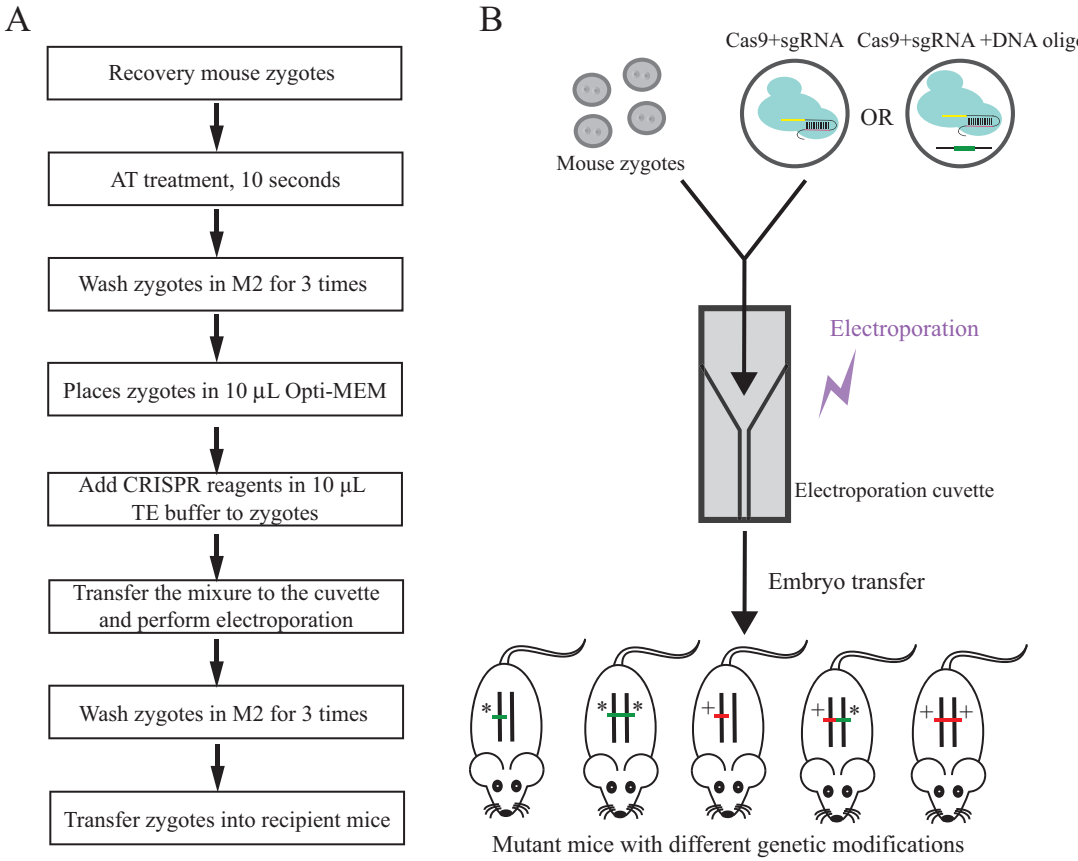

Fig. 1 ZEN Enables High-Throughput generation of genetically modified Mice. (**a**) Flow chart of ZEN method. (**b**) The process of generating genetically modified mice using ZEN. *Asterisk* represents precise modification through HDR, and *plus* indicates indel mutations resulted from NHEJ

Compared to microinjection, ZEN is easy to set up, less laborious, high-throughput, and has a gene-editing efficiency similar to microinjection [6, 7]. After we published our results, another group also reported that delivery of Cas9 RNP (Cas9:sgRNA ribonucleoprotein) through electroporation is efficient to generate genetic modification in the mouse genome, confirming the generality of this method [8].

In this protocol paper, we describe this method in detail.

2 Materials and Equipment

2.1 Mouse Embryo Preparation

1. Donor female mice (various strains, 12–15 g or 8–10 weeks, The Jackson Laboratory).
2. Stud male mice (various strains, 12–24 weeks, The Jackson Laboratory).
3. PMSG (pregnant mare's serum gonadotropin, ProSpec).

4. hCG (human Chorionic Gonadotropin, ProSpec).
5. Hyaluronidase.
6. M2 medium.
7. K-RVCL-50 Medium (COOK Medical).
8. Mineral oil (Sigma-Aldrich).
9. Needles: 25-gauge 5/8″; 30-gauge 1/2″ needle.
10. One microliter syringe.
11. Mouth micropipette.
12. Microdissecting instruments.
13. Tissue culture dishes: 60 mm; 100 mm.
14. MINC Benchtop Incubator (COOK Medical).
15. SteREO Discovery. V8 Microscope (Zeiss).

2.2 sgRNA Synthesis

1. Two universal primers and primers with various sgRNA sequences (Table 1) (Integrated DNA Technologies).
2. PrimeSTAR GXL DNA Polymerase (Clontech).
3. RNase-free PCR tubes.
4. RNase-free microfuge tubes (1.5 mL).
5. RNase AWAY™ Surface Decontaminant (Thermo Fisher Scientific).
6. MEGAshortscript T7 kit (Thermo Fisher Scientific).
7. QIAquick PCR Purification Kit (Qiagen).
8. SeaKem LE Agarose (Lonza).
9. 10× TAE buffer-4 L (Lonza).
10. GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific).
11. 6× DNA loading dye.
12. Thermo cycler.
13. Electrophoresis equipment and supplies.
14. Manual Gel Documentation Systems (InGenius3, Syngene).
15. NanoDrop 2000 (Thermo Fisher Scientific).
16. Centrifuge.

Table 1
Primer information

Name	Sequence (5′ to 3′)
Universal Rev. Primer 1	Aaaaaagcaccgactcggtgccacttttcaagttgataacggactagccttatttaaacttgc tatgctgtttccagcatagctcttaaac
Universal Rev. Primer 2	aaaagcaccgactcggtgcc
Forward Primer	taatacgactcactatag-gRNA sequence-gtttAagagctatgctggaaac

2.3 Electroporation

1. Ultramer DNA oligos (Integrated DNA Technologies).
2. Cas9 protein (PNA Bio).
3. M2 medium.
4. OptiMEM reduced serum medium (Gibco).
5. Acidic Tyrode's solution (Sigma-Aldrich).
6. RNase-free microfuge tubes (1.5 mL).
7. Savant SpeedVac concentrator (Thermo Fisher Scientific).
8. ECM830 Square Wave Electroporation System (BTX Harvard Apparatus).
9. MINC Benchtop Incubator (COOK Medical).
10. SteREO Discovery.V8 Microscope (Zeiss).
11. Centrifuge.

2.4 Sample Collection and Genotyping

1. Mouse ear punch.
2. Scissors.
3. PCR tubes or 96 plates.
4. Genotyping primers (Integrated DNA Technologies).
5. PrimeSTAR GXL DNA Polymerase (Clontech).
6. Zero Blunt TOPO® PCR Cloning Kit (Invitrogen).
7. SeaKem LE Agarose (Lonza).
8. 10× TAE buffer-4 L (Lonza).
9. GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific).
10. 6× DNA-loading dye.
11. Thermo cycler.
12. Electrophoresis equipment and supplies.
13. Manual Gel Documentation Systems (InGenius3, Syngene).
14. LB plates with specific antibiotics.

3 Methods

3.1 Zygote Isolation and Grading

All animal work must be approved by the local Institutional Animal Care and Use Committee and adhered to the standards of Guide for the Care and Use of Laboratory Animals set forth by the NIH. The embryos for electroporation can either be produced through natural breeding or from IVF (in vitro fertilization). Embryos from natural breeding usually have better quality, while IVF can produce large quantities of embryos. Here, we briefly introduce how embryos are produced through natural breeding [6, 7] (*see* **Note 1**).

3.1.1 Superovulation

1. Order female mice (6–8 weeks) of desired strain (*see* **Note 2**).
2. Inject mice intraperitoneally (i.p.) with 2.5 IU or 5 IU PMSG.
3. Inject mice i.p. with 2.5 IU or 5 IU hCG 46–50 h after administration of PMSG.

3.1.2 Mating

Immediately post administration of hCG, mate the female mice with stud males at a 1:1 ratio. The next day (22 h later), check for the presence of a copulation plug.

3.1.3 Harvest Embryos

1. Euthanize female mice with a copulation plug, and open the peritoneal cavity using dissecting instruments.
2. Remove the oviducts from the female mice.
3. Transfer the oviducts into M2 medium containing hyaluronidase (0.3 mg/mL).
4. Release the oocyte clutch by puncturing the ampulla with a pair of microdissecting forceps and allow them to incubate in the M2 medium containing hyaluronidase until the cumulus cells fall off and the embryos are disaggregated.
5. Wash the embryos with warm M2.

3.1.4 Embryos Grading

1. Transfer embryos to a new drop of fresh M2 medium and grade for fertilization and viability by examining the presence of the two pronuclei and integrity of the membranes enclosing the embryo.
2. Pass the graded zygotes through two washes of fresh M2 medium and then place in microdrops of K-RVCL-50 medium that have been equilibrated under mineral oil in an incubator (37 °C, 5% CO_2/5% O_2/Nitrogen).

3.2 sgRNA Preparation

3.2.1 sgRNA Sequence Selection

Several sgRNA designing programs are freely available online. The consideration of sgRNA design and features of different programs are reviewed elsewhere [9, 10]. In this protocol, we use CRISPR Design (http://crispr.mit.edu/) as an example.

1. Find the target region of the gene of interest. The location of the target region is dependent on the aim of the project. For example, if KO is required, the region should be located to the downstream of the start codon but relatively to the 5′ region of the gene. More detailed design principles are described in Qin et al. [10].
2. Copy about 100 nt within this region and paste into the sequence window on CRISPR Design webpage. Select the target genome, which is the model organism you are using and submit the mission. Select the available guides with the highest score indicating the lowest potential off-target efficiency.
3. Use the selected sgRNA sequences to design the PCR primers (Table 1).

3.2.2 PCR Amplification and PCR Products Purification

In this protocol, three primers are used in the PCR amplification: Two universal primers and one unique primer with specific sgRNA sequences are listed in Table 1. The T7 promoter sequence was added in front of the sgRNA sequence.

1. Set up the PCR reaction (50 μL):
   First, prepare the master mix:

Reagent	Volume (μL)
10× PCR reaction buffer	10
dNTP	4
Universal Rev. Primer 1 (10 μM)	3
Universal Rev. Primer 2 (10 μM)	3
PrimeStar GXL taq	1
H_2O (Molecular Grade)	26

   Aliquot 47 μL of master mix per reaction and add 3 μL of sgRNA forward primer (10 μM) to each reaction. Mix well and load the PCR reactions to a thermo cycler.

2. PCR cycles:
   (1) 98 °C, 2 min; (2) 98 °C, 30 s; (3) 60 °C, 30 s; (4) 68 °C, 1 min; to **step 2**), 30 cycles; (5) 68 °C, 5 min; (6) 4 °C, hold.

3. Purify PCR products using QIAQuick PCR purification kit according to the manufacturer's instructions. Avoid all RNase contamination in subsequent steps. Elute PCR products with 30–50 μL RNase-free water. The final concentration of PCR products should be more than 50 ng/μL. Purified PCR products are the templates for the in vitro transcription (IVT) of sgRNAs.

3.2.3 sgRNA Synthesis

1. Set up sgRNA IVT reactions:

Reagent	Volume (μL)
T7 10x Reaction Buffer	2
ATP	2
CTP	2
GTP	2
UTP	2
T7 Enzyme Mix	2

   Aliquot 12 μL of master mix per reaction and add 8 μL of purified PCR product. Mix well by pipetting.

2. Incubate for 4 h at 37 °C.

3. Add 1 μL of Turbo DNase (from MEGAshortscript kit) to each reaction and incubate for 15 min at 37 °C to degrade the template DNA.

3.2.4 sgRNA Purification

Purify sgRNAs by precipitation and rehydration:

1. Transfer 21 μL reaction to a new 1.5 mL RNase-free Eppendorf tube.
2. Add 115 μL nuclease-free water and 15 μL NH_4OAc stop solution from the MEGAshortscript kit to each reaction and mix well.
3. Add 300 μL of 100% EtOH and mix thoroughly.
4. Chill the mixture at −80 °C for at least 20 min.
5. Centrifuge at full speed (>10,000 × *g*) at 4 °C for 15 min.
6. Remove the supernatant carefully and add 1 mL of 70% EtOH to wash the RNA pellet.
7. Centrifuge at full speed (>10,000 × *g*) at 4 °C for 5 min.
8. Remove the supernatant and air-dry the pellet.
9. Resuspend the pelleted RNA in 50 μL of nuclease-free water or Ambion's Elution Solution.
10. Quantify the concentration of sgRNA using NanoDrop. The 260/280 ratio at 2.0 or above is indicative of a clean RNA preparation. The sgRNA can be diluted to 2 μg/μL and frozen in −80 °C for future use.

    Alternatively, sRNA can be purified using MEGAclear kit following manufacturer's instruction.

3.2.5 sgRNA Quality Control

1. Transfer 2 μL of final sgRNA product (around 1–2 μg/μL) to a new PCR tube free of RNase.
2. Add 2 μL distilled RNase-free water.
3. Add 4 μL Gel Loading Buffer II from the Megashortscript kit.
4. Mix well and incubate at 65 °C for 10 min.
5. Directly put the samples on ice for at least 2 min.
6. Load the samples and 3 μL 1Kb plus DNA marker to 1% agarose gel.
7. Electrophorese the gel at 120 V until the bromophenol blue reaches the middle part of the gel.
8. Check the gel under UV and take images. There should be one single and sharp band for each sgRNA product at around 200 bp.

3.3 Electroporation Mixture Preparation

1. Prepare the reagent at 2× concentration in 10 μL volume in TE buffer:

   Cas9 protein: 500 ng/μL; sgRNA: 600 ng/μL; DNA: oligo donors 1 μg/μL.

2. Incubate at 37 °C for 15–30 min.
3. Put the samples on ice and perform electroporation immediately (*see* **Note 3** and **4**).

3.4 Zygote Electroporation and Embryo Transfer (see Note 5)

1. Place the mouse zygotes in the acidic Tyrode's solution for 10 s (IVF embryos do not need this treatment, since their zona pellucida has been weakened during IVF).
2. Remove the embryos and wash through three drops of 100 μL pre-warmed M2 media.
3. Place the embryos in 10 μL drop of Opti-MEM media that has been pre-warmed and equilibrated. For each sample, up to 150 zygotes can be electroporated together, and we routinely use 60 zygotes. Depending on the skill level of the personnel handling the embryos, different numbers of zygotes can be used.
4. Add 10 μL of CRISPR-Cas9 reagents, consisting of the Cas9 protein, sgRNA, and donor oligonucleotide reconstituted in TE buffer (pH 7.5), to the embryos in Opti-MEM medium.
5. Pipet up and down a few times gently, pick up the 20 μL of embryos suspended in CRISPR-Cas9 reagents with a 20 μL pipet tip, position the pipet tip between electrodes of the cuvette, and deposit the contents into the chamber of a 1 mm electroporation cuvette.
6. Load the cuvette to the electroporator (We use ECM830 Square Wave Electroporation System). The electroporation setting is the same for all the experiments using 1 ms pulse duration and two pulses with 100 ms pulse interval at 30 V.
7. Press the start button. One-time electroporation is quite efficient for generating mouse models we tested, but multiple electroporation can be used to further improve the efficiency by pressing start button four to six times, with 3 s interval.
8. Following the electroporation, deposit pre-warmed and pre-equilibrated 100 μL M2 medium into the cuvette using a sterile plastic pipette (comes with the cuvette).
9. Remove the medium containing zygotes from the cuvette and place into a Petri dish.
10. Rinse the cuvette with additional 100 μL of M2 and add to the embryos in Petri dish.
11. Transfer the zygotes to the culture dish and keep them in culture in a MINC benchtop incubator (COOK; 37 °C, 5% CO_2).
12. The embryos will be transferred into pseudopregnant female mice (CByB6F1/J).
13. Embryos at pronuclear stage will be transferred into each recipient mouse, following the standard embryo transfer protocol [11].
14. Alternatively, these embryos can be kept in culture and develop into blastocysts for genotyping.

3.5 Blastocysts Collection and Genomic DNA Extraction

It is useful to genotype the blastocysts in vitro developed from electroporated zygotes to evaluate the gene-editing efficiency before generating live born mice. Here, we describe the blastocysts genotyping method.

1. Use thin-wall strip PCR tubes with individual attached caps. Open the caps and put the PCR tubes under the inverted microscope.
2. Make several drops of M2 medium.
3. Transfer blastocysts in the culture dish to a drop of warm M2.
4. Wash the blastocysts in the drops of M2 medium to get rid of the mineral oil.
5. Load 8–16 blastocysts into the mouth micropipette.
6. Put one embryo in each PCR tube under the inverted microscope, keeping the volume of the liquid to the minimum.
7. Prepare blastocysts genomic DNA extraction buffer (10 mM Tris–HCl, pH 8.0; 2 mM EDTA; 2.5% Tween-20, 2.5% Triton-X 100; 100 μg/mL Proteinase K).
8. Add 6 μL of the extraction buffer to each PCR tube.
9. Perform the extraction reaction in a thermo cycler: 55 °C, 1 h; 95 °C, 10 min; RT, hold.
10. The extracted DNA can be stored in −20 °C or directly used for genotyping.

3.6 Mouse Tissue Collection and Genotyping

Pups at day 12 or after day 12 are ready for tissue collection. Either ear notch or tail tip can be used for genotyping. Ear notch and tail tip sampling can be done following the standard procedure.

3.6.1 Mouse Tissue Collection

3.6.2 Extraction of Genomic DNA from Mouse Tail Tip or Ear Notch

1. Put 2 mm tail tip or ear punch in a PCR tube or 96-well plate. Spin to collect the samples to the bottom of the wells.
2. Add 50 μL 50 mM NaOH and quick spin to make sure tissue samples are in the solution.
3. Perform the extraction reaction in a thermo cycler: 98 °C, 1 h; 25 °C, hold.
4. Add 60 μL neutralization buffer (167 mM Tris–HCl, pH 8.0, 42 mM HCl) to neutralize the reaction and mix well. This mixture will serve as the template for the following PCR amplification.

3.6.3 PCR Reaction and DNA Electrophoresis

1. Set up the PCR reaction:

Reagent	Volume (μL)
10× PCR reaction buffer	5
dNTP	2
Universal Rev. Primer 1	1.5
Universal Rev. Primer 2	1.5
PrimeStar GXL taq	0.5
H_2O (Molecular Grade)	13.5

Add 1 μL of genomic DNA sample in 25 μL reaction and mix well. The remaining DNA samples can be kept at 4 °C for a few weeks.

2. PCR cycles:
   (1) 98 °C, 2 min; (2) 98 °C, 30 s; (3) 60 °C, 30 s; (4) 68 °C, 1 min; to **step (2)**, 34 cycles; (5) 68 °C, 5 min; (6) 4 °C, hold.
3. Transfer 2 μL of the PCR reaction to a new tube.
4. Add 3 μL of water.
5. Add 1 μL of 6× loading dye and mix well.
6. Load the samples and 3 μL 1Kb plus DNA marker to 1% agarose gel.
7. Electrophorese the gel at 120 V until the bromophenol blue reaches the bottom of the gel.

3.6.4 Sanger Sequencing and Genotyping Strategies

1. Prepare required PCR products and desired primers following the required protocol for Sanger sequencing. Send the PCR products for sequencing. TIDE can be used to analyze different alleles existing in these PCR products [12].
2. T7E1 assay or Surveyor assay can be performed to confirm insertion or deletion (indel) formation following standard procedures.
3. If an enzymatic site (Knock-in experiment) is inserted, digest 4 μL PCR products with specific restriction enzymes. Run an agarose gel to confirm the insertion.

3.6.5 Sequencing Trace Analysis Using TIDE Online Software

1. Open the webpage of TIDE online analysis tool following the link: https://tide-calculator.nki.nl/.
2. Type in sample name and gRNA sequence.
3. Load control sequencing traces and sample sequencing traces (SCF or ABI chromatogram files).
4. Tick advanced settings and click update view. The alignment window, decomposition window, and indel size range can be adjusted.
5. Click update view.
6. The indels and the according frequency will be shown. If a small DNA insertion is expected, adjust the indel size range to

include the expected insert size. If the desired insert exists in the sample, there will be a peak at the expected insert size. The frequency of the alleles with the insert will be shown.

3.6.6 TOPO Blunt-End Cloning and Sanger Sequencing

To confirm that the genomic modifications are precise and correct, the PCR product needs to be subcloned into TOPO blunt-end cloning vectors.

1. Perform the TOPO cloning using Zero Blunt TOPO® PCR Cloning Kit following the manufacturer's protocol.
2. Pick and expand the single clones.
3. Purify the plasmids.
4. Sequence the insert by Sanger sequencing.

3.6.7 Southern Blot

The genomic DNA can also be used for southern analysis following the standard procedure.

4 Notes

1. Surgical training in the Jackson Laboratory. There is a workshop on Assisted Reproductive Technologies (ARTs) in the laboratory mouse every year in the Jackson Laboratory. We encourage people to get proper training from these useful workshops.
2. Mouse strain selection. We used C57B6/J and C57B6/NJ in our experiments. The targeting efficiency and the survival rate of the pups are both high. In addition, NSG mice can also be modified using this technology, as it is a more mild treatment for the embryos compared to the microinjection. This is very useful for generating genetic modifications in mouse strains that are sensitive to microinjection.
3. Cas9 RNP preparation. The Cas9 protein can be diluted with RNase-free water or TE, and kept at −80 °C. Multiple freeze-thaw cycles should be avoided. The electroporation solution can also be frozen in −80 °C before incubation at 37 °C. Thaw the frozen electroporation solution and incubate in 37 °C for 15–30 min before performing electroporation.
4. Cas9 mRNA can also be used in the ZEN technology. The final concentration of Cas9 mRNA will be 600 ng/μL in the electroporation solution. Please note that compared with Cas9 RNP, Cas9 mRNA has much lower targeting efficiency for several target genes we tested.
5. ZEN technology can be used to generate indel mutations, large DNA segment deletions, specific nucleotide changes, and small DNA fragment insertions. DNA segment deletion can be generated using two sgRNAs simultaneously. A donor

oligonucleotide can be used to generate precise nucleotides changes or small tag insertion (3× FLAG, His, V5, loxP, etc.). The oligonucleotide is designed such that the mutation is flanked by 40–60 nt long homology arms on each side. The intended mutation should disrupt (for tag or loxP insertion models) or replace (for point mutation incorporation models) the PAM or the guide sequence, particularly the PAM proximal sequences. Order the oligonucleotide from a vendor, considering using PAGE or HPLC to enrich for the full-length oligonucleotide.

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