Product Manual

RAPAd® miRNA Adenoviral Expression System

Catalog Number

VPK-253	1 kit

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

MicroRNAs (miRNAs) are 18–24 nucleotide RNA molecules that regulate the stability or translational efficiency of target mRNAs. These regulatory RNAs function by acting as sequence-specific guides which recruit a large protein complex known as the RNA-induced silencing complex (RISC) to target mRNAs which are subsequently silenced. Diverse functions have been attributed to miRNAs including the regulation of cellular differentiation, proliferation, and apoptosis. Moreover, significant evidence has accumulated implicating a fundamental role for miRNAs in the development of cancer. miRNAs are initially transcribed as long precursor transcripts known as primary microRNAs (pri-miRNAs). Within these transcripts, the mature miRNA sequences are found in ~60–80 nucleotide hairpin structures. Mature miRNAs are generated from pri-miRNAs by sequential processing (Figure 1). Pri-miRNAs are initially recognized in the nucleus by the microprocessor complex which includes as core components the RNase-III enzyme Drosha and its obligate partner DGCR8. This complex excises the hairpin structure containing the mature miRNA sequence. The liberated hairpins, referred to as precursor miRNAs (pre-miRNAs), are recognized by the nuclear export factor exportin 5 which transports them to the cytoplasm. There, the RNase-III enzyme Dicer performs a second cleavage to generate a double-stranded 18–24 nucleotide RNA molecule. The RISC then associates with this RNA duplex and unwinds it. Generally, only one strand is stably incorporated into the RISC; the other is discarded and rapidly degraded. miRNAs guide the RISC to target messages that are subsequently cleaved or translationally silenced (Figure 1).

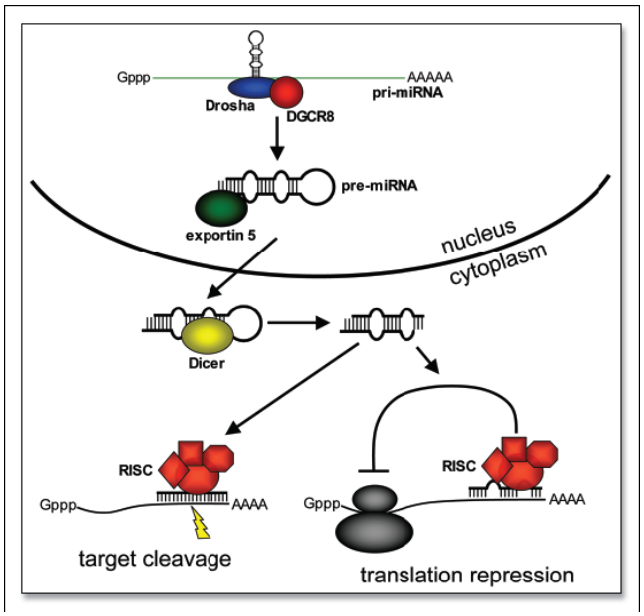


Figure 1. miRNA Biogenesis and function

Recombinant adenoviruses have tremendous potential in both research and therapeutic applications. There are numerous advantages they provide when introducing genetic material into host cells. The permissive host cell range is very wide. The virus has been used to infect many mammalian cell types (both replicative and non-replicative) for high expression of the recombinant protein. Recombinant adenoviruses are especially useful for gene transfer and protein expression in cell lines that have low transfection efficiency with liposome. After entering cells, the virus remains epichromosomal (i.e. does not integrate into the host chromosome so does not activate or inactivate host genes). Recently, recombinant adenoviruses have been used to deliver RNAi into cells.

Cell Biolabs' RAPAd® Adenoviral Expression System provides a much faster and safer method to generate RCA-free recombinant adenovirus at high titer. The RAPAd® system uses a novel Ad backbone devoid of the left-hand ITR, the packaging signal and E1 sequences. There is no need to perform the bacterial *in vitro* homologous recombination (pAdEasy method), and also the multiple plaque isolations (standard homologous recombination method in packaging cell line). The RAPAd® system allows for generation of a recombinant virus within 2 weeks and the virus produced contained virtually no contaminating E1a sequences or replication-competent virus (RCA).

Cell Biolabs' RAPAd® miRNA Adenoviral Expression System is designed to rapidly produce recombinant adenovirus that expresses an individual miRNA precursor in its native context while preserving putative hairpin structures to ensure biologically relevant interactions with endogenous processing machinery and regulatory partners, leading to properly cleaved microRNAs. Individual miRNA precursor from any species can be cloned between BamHI and Nhe I sites (Figure 2).

RAPAd® miRNA Adenoviral Expression System contains the following unique features:

- **miRNA Processing** – miRNA stem loop precursor in its native context is cloned between BamHI and Nhe I sites. To preserve the putative hairpin structure and proper endogenous processing, miRNA stem loop sequence is flanked by its native intron sequence.
- **EF-1α Promoter -** ensures a high level of expression in mammalian cells
- **GFP-Puro Fusion Marker -** to monitor cells positive for expression and stable selection with either GFP or puromycin resistance.

Related Products

1. AD-100: 293AD Cell Line
2. AD-200: ViraDuctin™ Adenovirus Transduction Reagent
3. VPK-090: ViraBind™ Lentivirus Concentration and Purification Kit
4. VPK-099: ViraBind™ Adenovirus Miniprep Kit
5. VPK-100: ViraBind™ Adenovirus Purification Kit
6. VPK-109: QuickTiter™ Adenovirus Titer Immunoassay Kit
7. VPK-110: QuickTiter™ Adenovirus Titer ELISA Kit
8. VPK-111: Rapid RCA Assay Kit
9. VPK-130: ViraBind™ Retrovirus Concentration and Purification Kit
10. VPK-250: RAPAd® Universal Adenoviral Expression System
11. VPK-251: RAPAd® RSV Adenoviral Expression System
12. VPK-254: RAPAd® CMV Adenoviral Bicistronic Expression System (GFP)

Kit Components

1. pacAd5 miR-GFP/Puro Shuttle Vector (Part No. 325301): One 40 µL vial at 0.25 mg/mL.
2. pacAd5 9.2-100 Vector (Part No. 325002): One 40 µL vial at 0.25 mg/mL.

3. pacAd5 CMV-GFP Control Vector (Part No. 325004): One 40 µL vial at 0.25 mg/mL.
4. pacAd5 CMV-ntLacZ Control Vector (Part No. 325202): One 40 µL vial at 0.25 mg/mL.

Materials Not Supplied

1. 293 cells: we recommend 293AD Cell Line (Cat.# AD-100) for high titer production of recombinant adenovirus.
2. 293 Cell Culture Medium
3. Transfection Reagents
4. PacI (New England Biolabs, Cat.# R0547L)

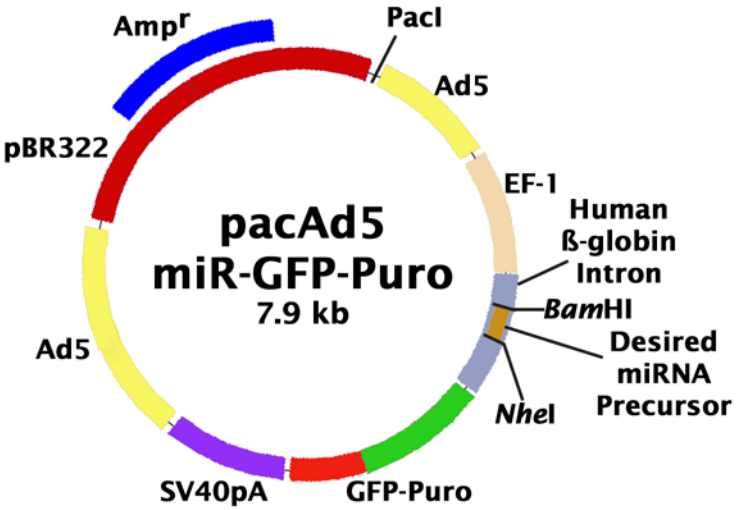
Storage

Upon receipt, store all kit components at -20ºC.

Safety Considerations

Remember that you will be working with samples containing infectious virus. Follow the recommended NIH guidelines for all materials containing BSL-2 organisms.

Vector Features



Multiple Cloning Sites:

```
GATTAGTTCTCGAGGATCCGACTGAAGTCGCTAGCTCGAGCTTTTGGA
          BamHI            NheI
```

Figure 2. pacAd5 miR-GFP/Puro Shuttle Vector (7900 bp, Ampicillin-resistant). Xho I Digestion: 26 bp + 660 bp + 7214 bp

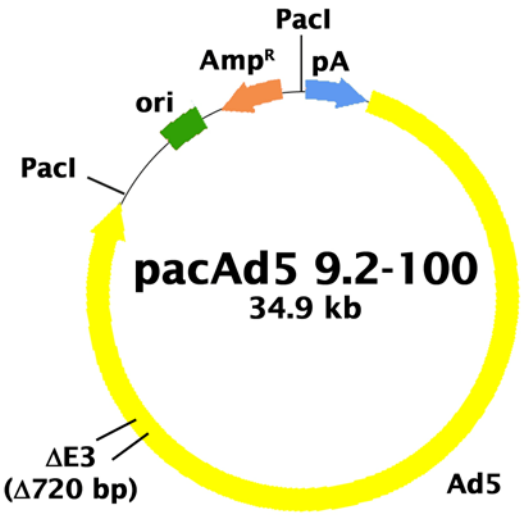


Figure 3. pacAd5 9.2-100 Vector (34947 bp, Ampicillin-resistant). The novel pacAd5 9.2-100 Ad backbone vector is devoid of the left-hand ITR, the packaging signal and E1 sequences.

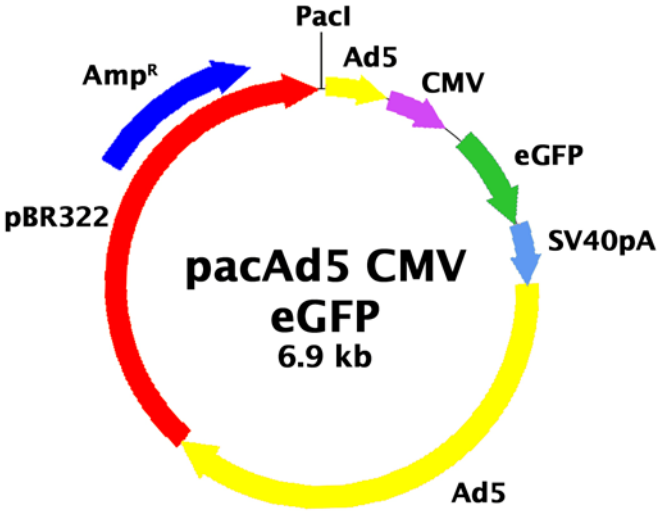


Figure 4. pacAd5 CMV-GFP Control Vector (6935 bp, Ampicillin-resistant).

pacAd5 CMV-GFP Features:

3-10:	PacI
16-368:	1-353 of Ad5
385-912:	CMV Promoter
992-1711:	GFP
1713-2160:	SV40 pA
2161-4615:	3328-5792 of Ad5
5867-6727:	β Lactamase

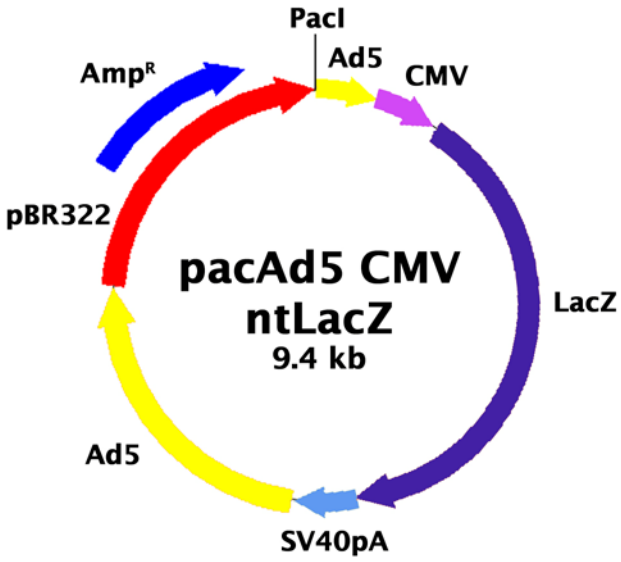


Figure 5. pacAd5 CMV-ntLacZ Control Vector (9278 bp, Ampicillin-resistant).

pacAd5 CMV-ntLacZ Features:

3-10:	PacI
16-368:	1-353 of Ad5
385-912:	CMV Promoter
1105-4148:	ntLacZ
4193-4640:	SV40 pA
4641-7095:	3328-5792 of Ad5
8347-9210:	β Lactamase

miRNA Precursor Cloning

All of our premade human amd mouse miRNA precursor clones in mammalian expression vectors (pEP-miR or pEPGP-miR) are based on the following design, and the resulting overexpression of the mature miRNA is confirmed by Northern blot or Real Time PCR. Here we use human let-7a-2 miRNA as an example:

1. Download desired miRNA stem loop sequence from Sanger's miRNA database:
http://microrna.sanger.ac.uk/sequences/

Homo sapiens let-7a-2 stem-loop structure

```
   uu   g   u             uagaa ua a
agg  gag uag agguuguauaguu     u  c u
|||  ||| ||| |||||||||||||     |  | c
ucc  uuc auc uccgacaugucaa     a  g a
   -u   g   c             --uag gg a
```

Homo sapiens let-7a-2 stem-loop sequence

AGGUUGAGGUAGUAGGUUGUAUAGUUUAGAAUUACAUCAAGGGAGAUAACUGUACAGCCUCCUAGCUUUCCU

2. Blast search miRNA stem loop sequence: http://blast.ncbi.nlm.nih.gov/Blast.cgi

```
>  ref|NT_033899.7|Hs11_34054  Homo sapiens chromosome 11 genomic contig,
reference assembly
Length=38509590

Query  1         AGGTTGAGGTAGTAGGTTGTATAGTTTAGAATTACATCAAGGGAGATAACTGTACAGCCT  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25579717  AGGTTGAGGTAGTAGGTTGTATAGTTTAGAATTACATCAAGGGAGATAACTGTACAGCCT
25579658

Query  61        CCTAGCTTTCCT  72
                 ||||||||||||
Sbjct  25579657  CCTAGCTTTCCT  25579646
```

3. PCR and Cloning:

1) Add 100 base native flank sequence to both upstream and downstream of the miRNA stem loop.

Human let-7a-2 miRNA precursor sequence including the 100 base flank sequences on both ends of the stem loop: let-7a-2 stem-loop sequence is underlined.

GCCCAAATAGGTGACAGCACGATGAATCATTATAAGACTAACTTGTAATTTCCCTGCTTAAGAA
ATGGTAGTTTTCCAGCCATTGTGACTGCATGCTCCCAGGTTGAGGTAGTAGGTTGTATAGTTTA
GAATTACATCAAGGGAGATAACTGTACAGCCTCCTAGCTTTCCTTGGGTCTTGCACTAAACAAC
ATGGTGAGAACGATCATGATTCCTCCAGGCCTTTTCTCCCTATGAAAGGTAAGATTGGGTACGA
TTATTTTATGGTATTT

2) Design PCR primer including BamHI site at forward primer with four extra bases and NheI site at reverse primer.

Forward PCR Primer: `tcga-ggatcc (BamHI)-21 nt`
Reverse PCR Primer: `tcga-gctagc (NheI)-21 nt`

For human let-7a-2 miRNA precursor:
Forward PCR Primer: `tcga-ggatcc-gcccaaataggtgacagcacg`
Reverse PCR Primer: `tcga-gctagc-aaataccataaaataatcgta`

3) PCR the miRNA precursor from genomic DNA and clone into the BamHI/NheI sites of the expression vector.

PCR Product of let-7a-2 precursor: let-7a-2 stem-loop sequence is underlined.

1 tcga**ggatcc** gcccaaatag gtgacagcac gatgaatcat tataagacta acttgtaatt

```
 61 tccctgctta agaaatggta gttttccagc cattgtgact gcatgctccc aggttgaggt
121 agtaggttgt atagtttaga attacatcaa gggagataac tgtacagcct cctagctttc
181 cttgggtctt gcactaaaca acatggtgag aacgatcatg attcctccag gccttttctc
241 cctatgaaag gtaagattgg gtacgattat tttatggtat ttgctagctc ga
```

4) Validate the insert by DNA sequencing.
   Forward Sequencing Primer: TTTGCACCATTCTAAAGAAT
   Reverse Sequencing Primer: AAACCTCTTACATCAGTTAC

Preparation of Recombinant Adenovirus

I. Vector Linearization with PacI

1. Digest a sufficient amount of the pacAd5 shuttle vector containing gene of interest and the pacAd5 9.2-100 Ad backbone vector with PacI.
2. Run 0.5 µg of each digested DNA and undigested DNA on a 0.8% agarose gel to confirm the completion of PacI digestion (For pacAd5 9.2-100, one band of ~33 kb and a second band of 2.0 kb).
3. Remove buffer and enzyme from the remainder of the restriction reactions by phenol extraction/ethanol precipitation or using a similar DNA purification kit.
4. Resuspend the DNA in sterile dH_2O. Store the digested DNA at –20°C.

II. Transfection

1. Seed 2 x 10^6 cells in a 60 mm culture dish without antibiotics one day before transfection.
2. After 16 to 24 hours, start transfection when the culture becomes 70-80% confluence. *Note: We suggest transfecting cells with FuGENE® Transfection Reagent (Roche Applied Science) or Lipofectamine™ Plus (Invitrogen). For example, 4 µg of pacAd5 shuttle vector and 1 µg of pacAd5 9.2-100 Ad backbone vector are mixed with 9 µL FuGENE® Transfection Reagent according to the manufacturer's recommendation. The mixed DNA- FuGENE® complex is added by dropwise into the culture media.*
3. Aspirate the media containing transfection reagent the next day and add 4 mL of complete culture medium.
4. After incubating for 7 days, check for the presence of plaques. If plate is ready for harvest, (>50% of cells lifted), then collect the Crude Viral Lysate. If not, feed the cells with 1 mL of complete culture medium, continue to incubate at 37°C with CO_2.
5. On day 10, check for the presence of plaques. If plate is ready for harvest, (>50% of cells lifted), then collect the Crude Viral Lysate. If not, feed the cells with 1 mL of complete culture medium, continue to incubate at 37°C with CO_2. Keep checking plate for the presence of plaques. Do not keep plate more than 15 days.

III. Harvesting the Crude Viral Lysate

1. Harvest adenovirus-containing cells by squirting cells off the plate with a 5 or 10 mL sterile serological pipette. Transfer cells and media to a sterile 15 ml tube. Scrape the cells into the medium with a cell lifter if necessary.
2. Release viruses from cells by three freeze/thaw cycles (10 minutes each in 37°C water bath and dry ice-methanol bath).
3. Centrifuge the cell lysate in a table-top centrifuge at 3000 rpm for 15 minutes at room temperature to pellet the cell debris.
4. Aliquot and store the Crude Viral Lysate (Initial Viral Stock) at -80°C.

IV. Amplification

Note: The following procedure is suggested for T75 flasks and may be optimized to suit individual needs.

1. Seed 3-5 x 10^6 cells in a T75 flask one day before infection.
2. Add 50% of the above Crude Viral Lysate to the culture. We recommend using a multiplicity of $\geq$0.5 PFU (plaque forming units) or enough viruses that cells demonstrate cytopathic effects (CPEs) within 48 hrs.
3. During 24 - 48 hr infection, examine the monolayer twice per day under the microscope for CPE. When CPE is nearly complete (i.e. most cells rounded but not yet detached from the flask), harvest cells by pipetting media up and down to wash the infected cells from the flask into the media.
4. Pool infected cells and medium. Pellet cells by centrifugation at 1000 g for 5 minutes. Remove supernatant, resuspend cell pellet in medium or in 10 mM Tris, pH 8.0, 100 mM NaCl. (0.25-0.5 mL per T75 flask).
5. Release the adenoviruses from the cell suspension with three freeze/thaw cycles. Centrifuge at 3000 g for 10 minutes to pellet the cell debris. Discard the pellet and save supernatant as viral stock.
6. The viral supernatant can be stored at -80°C or immediately purified or titered.

Example of Results

The following figures demonstrate typical results of generating recombinant adenovirus. One should use the data below for reference only. This data should not be used to interpret actual results.

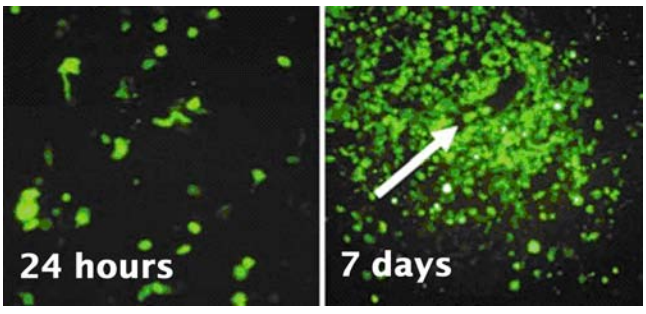


Figure 6. Generation of recombinant adenovirus using the RAPAd® Adenoviral Expression System. 293 cells were transfected with PacI linearized pacAd5 CMV-GFP vector and pacAd5 9.2-100 vector. Plates were examined for the presence of viral foci under inverted fluorescence microscope.

Appendix

pacAd5 miR-GFP-Puro Shuttle Vector Features:

3-10:	PacI
16-368:	1-353 of Ad5
409 ~ 804:	EF-1α Promoter
837 ~ 1327:	human β-globin intron
1053 ~ 1058:	BamHI
1069 ~ 1074	NheI
1355 ~ 2677:	GFP-Puro Fusion (GFP: 1355 ~ 2071; Puro: 2078 ~ 2677)
2697~ 2938:	SV40 pA
3120 ~ 5584:	3328-5792 of Ad5
6832 ~ 7692:	β Lactamase

pacAd5 miR-GFP/Puro Shuttle Vector Sequence:

```
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CTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAAT
CTAAAGTATATATGAGTAAACTTGGTCTGACAG**TTA**CCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGA
CTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAG
CAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAG
TTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGA
TCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCA
TGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTAT
GCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGA
AAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGT
GAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACT**CAT**ACTCTTCCTTTTTCAATATTATTGAAGCATTTA
TCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC
TAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAA

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