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I. Description

LA PCR *in vitro* Cloning Kit is an improved cloning system to specifically amplify the long unknown region on cDNA and genomic DNA based on the advantage of LA PCR Technology with the improved enzyme, *TaKaRa LA Taq*, and by using Cassette (double strand synthetic oligonucleotide with the restriction site at one end) and Cassette Primer. As LA PCR Technology provides the high fidelity, this kit reduces the occurance of mutation when cloning. By this kit, target long DNA fragment, such as genome DNA, can be easily obtained without constructing of the library nor screening.

II. Kit Components (10 reactions: 50 μl PCR reaction)

1. *Sau*3A I Cassette (200 ng/μl) .... 25 μl
   *Sau*3A I is a 4-base cutter restriction enzyme.When genome DNA is digested by *Sau3*A I, more fragments are generated than by other restriction enzymes.So, the concentration of *Sau*3A I cassette is higher than other cassettes. *Sau*3A I cassette is used after being diluted by 10-fold at control reaction. At general reaction, use this cassette without diluting.
2. *Eco*R I Cassette (20 ng/μl) .... 25 μl
3. *Hin*d III Cassette (20 ng/μl) .... 25 μl
4. *Pst* I Cassette (20 ng/μl) .... 25 μl
5. *Sal* I Cassette (20 ng/μl) .... 25 μl
6. *Xba* I Cassette (20 ng/μl) .... 25 μl
7. Cassette Primer C1 (10 pmol/μl) .... 20 μl
8. Cassette Primer C2 (10 pmol/μl) .... 20 μl
9. Ligation Solution I* .... 150 μl
10. Ligation Solution II* .... 75 μl
11. *TaKaRa LA Taq* (5 units/μl) .... 10 μl
12. 10X LA Buffer II (25 mM Mg^{2+} plus) .... 100 μl
13. dNTP Mixture (each. 2.5 mM) .... 160 μl
14. Control DNA fragment
    [human genomic DNA (HL60) *Bam*H I-digested fragment] (100 ng/μl) .... 25 μl
15. Control Specific Primer CS-1 (10 pmol/μl) .... 10 μl
16. Control Specific Primer CS-2 (10 pmol/μl) .... 10 μl

* Ligation Solution I and II are the same as the components of DNA Ligation Kit Ver.2.1 (# 6022).

Sequences of Control Primers
Control Specific Primer CS-1
5'-ATAGTGGAATGAAGGTTCATTTTTCATTCTCACAA-3'

Control Specific Primer CS-2
5'-TGATAGGCACTGACTCTCTGTCCCTTGGGCTGTTT-3'

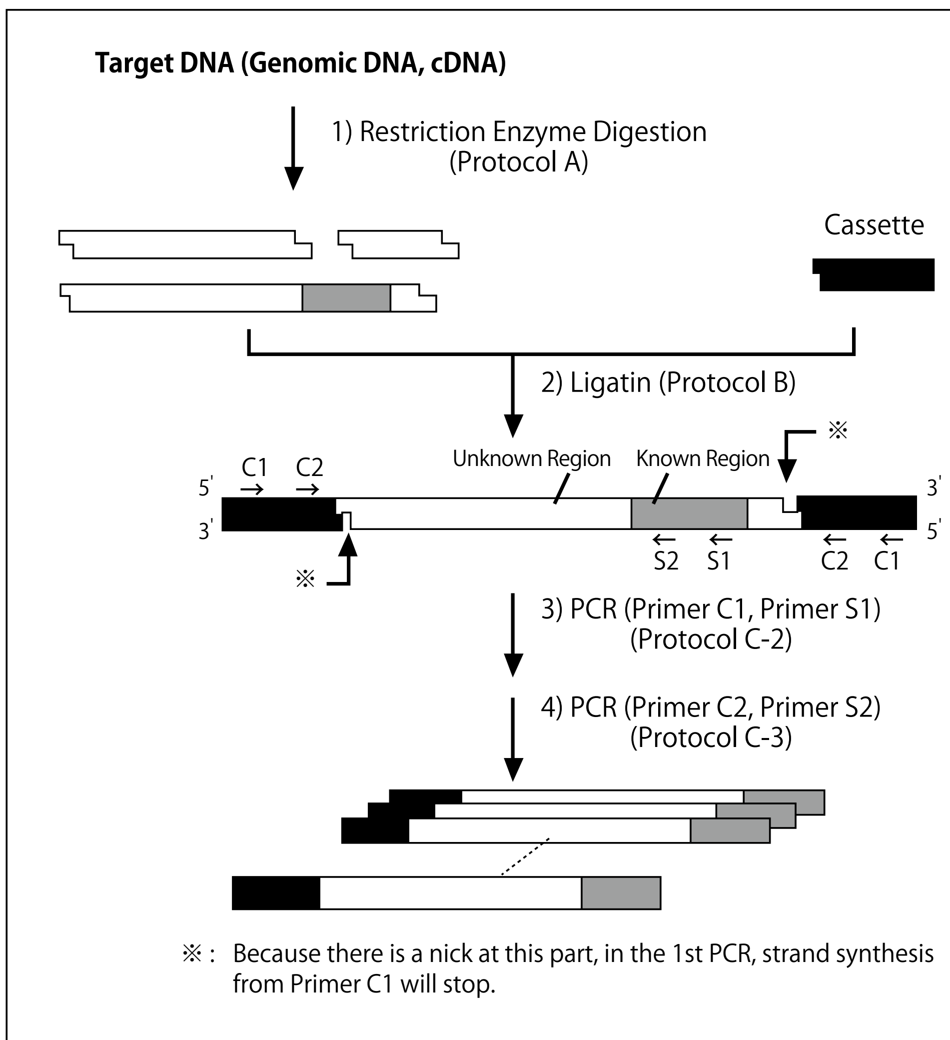
III. Reagents not Supplied in the Kit

Primers S1 and S2 complementary to the known region are not included.
Customer shall prepare his own specific Primers S1 and S2.

IV. Storage -20℃

V. Principle

1) Target DNA is completely digested by an appropriate restriction enzyme.
2) Ligate the Cassette with the restriction site of corresponding restriction enzyme.
3) Perform the 1st PCR by using Cassette Primer C1 and Primer S1 of the known region.
4) Perform the 2nd PCR by using primers designed for inner sequences,Cassette Primer C2 and Primer S2 of the known region. Target DNA will be specifically amplified.



Since the 5'-end of Cassette is not phosphorylated, a nick will occur within the ligation site of the 5'-end of Cassette and 3'-end of the target DNA. In the 1st PCR, strand synthesis from Primer C1 will stop at the nick and prevent the non-specific amplification. Only the strand synthesized from Primer S1 will be utilized as the template of DNA synthesis of the complementary strand from Primer C1. And the 2nd PCR using inside primers (Primer C2, S2) will amplify in more specific and in higher yield.
In case the partial amino acids sequence of the target protein is determined, mixed primer designed from the information may be used instead of Primer S1, S2 for cDNA cloning of the protein.

VI. Protocol

(1) General Reaction

A. Digestion of DNA with restriction enzyme

1) Prepare the mixture shown below, and incubate at an appropriate temperature for 3 - 5 hours.

DNA sample	5 μg
Restriction enzyme *	50 units *
10X Buffer for restriction enzyme	5 μl
Sterilized distilled water	up to 50 μl

*: The units of restriction enzyme necessary for the complete digestion of DNA depends on the purity of the sample DNA. 10 units of restriction enzyme for 1 μg DNA is recommended as the standard amounts for complete digestion.

2) Collect DNA by ethanol precipitation and dissolve the precipitate in 10 μl sterilized distilled water.

B. Ligation reaction

1) Prepare the reaction mixture shown below.

DNA sample prepareed at A	5 μl
Cassette *	2.5 μl
Ligation Solution I	15 μl
Ligation Solution II	7.5 μl

*: Use 10 times diluted *Sau*3A I Cassette with sterilized distilled water when ligating with DNA sample digested by 6-base restriction enzymes (*Bam*H I, *Bgl* II, *Fba* I, *Mfl* I).

2) Collect DNA by ethanol precipitation and dissolve the precipitate in 5 μl sterilized distilled water.

C. PCR

1) Add 1 μl of sample DNA solution prepared at B-2) to 33.5 μl of sterilized distilled water, and heat at 94℃ for 10 minutes.

2) Perform the 1st PCR shown below.

C-1) DNA solution	34.5 μl
10X LA Buffer II (Mg^{2+} plus)	5 μl
TaKaRa LA Taq	0.5 μl
dNTP Mixture	8 μl
Primer C1	1 μl
Primer S1	1 μl

In case of < 4 kb

94℃	30 sec.	30 cycles
55℃	30 sec.	
72℃	4 min.*	

*: Set 1 min. when amplification size is < 1 kb.

In case of ≧ 4 kb

96 - 98℃	10 - 20 sec.	30 cycles
68℃	15 min.	

3) Take 1 μl of 1 - 10,000 times diluted 1st PCR product with sterilized distilled water and perform the 2nd PCR shown below.

Diluted C-2) 1st PCR reactant	1 μl
10X LA Buffer II (Mg^{2+} plus)	5 μl
TaKaRa LA Taq	0.5 μl
dNTP Mixture	8 μl
Primer C2	1 μl
Primer S2	1 μl
distilled sterilized water	33.5 μl

In case of < 4 kb

94℃	30 sec.	30 cycles
55℃	30 sec.	
72℃	4 min.*	

*: Set 1 min. when amplification size is < 1 kb.

In case of ≧ 4 kb

96 - 98℃	10 - 20 sec.	30 cycles
68℃	15 min.	

4) Confirm the amplified DNA fragment by agarose gel electrophoresis.

NOTE : Because each cassette in this kit contains T7 promoter sequence, PCR products having T7 promoter are obtained by 1st PCR and 2nd PCR.

(2) Control Reaction

A. Ligation reaction

1) Prepare the following mixture for control reaction.

Control DNA fragment	5 μl
*Sau*3A I cassette (X 10 diluted) *	2.5 μl
Ligation Solution I	15 μl
Ligation Solution II	7.5 μl
	30 μl

*: As Control DNA fragment is *Bam*H I-digested fragment of human genomic DNA (HL 60), use *Sau*3A I cassette after being diluted by 10-fold with sterilized distilled water.

2) Collect DNA by ethanol precipitation and dissolve the precipitate in 5 μl sterilized distilled water.

B. PCR

1) Add 1 μl of DNA solution prepared at A-2) to 33.5 μl of distilled sterilized water, and heat at 94℃ for 10 minutes.

2) Perform the 1st PCR shown below.

B-1) DNA solution	34.5 μl
10X LA Buffer II (Mg^{2+} plus)	5 μl
TaKaRa LA Taq	0.5 μl
dNTP Mixture	8 μl
Primer C1	1 μl
Primer CS-1	1 μl

94℃ 30 sec.
55℃ 30 sec. 30 cycles
72℃ 30 sec.

3) Take 1 μl of 1 - 10,000 times diluted 1st PCR product with sterilized distilled water and perform the 2nd PCR shown below.

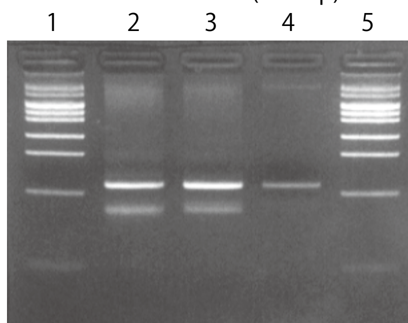
Diluted 1st PCR reactant	1 μl
10X LA Buffer II (Mg^{2+} plus)	5 μl
TaKaRa LA Taq	0.5 μl
dNTP Mixture	8 μl
Primer C2	1 μl
Primer CS-2	1 μl
Sterilized distilled water	33.5 μl

94℃ 30 sec.
55℃ 30 sec. 30 cycles
72℃ 30 sec.

4) Amplified fragment of 288 bp will be confirmed by the gel electrophoresis.

Control Reaction (288 bp)

1 2 3 4 5



Lane 1 : pHY Marker
2 : 1st. PCR reactant 1 μl
3 : 1st. PCR reactant X 10 diluted 1 μl
4 : 1st. PCR reactant X 100 diluted 1 μl
5 : pHY Marker

3% NuSieve® 3 : 1 Agarose gel.
applied volume : 5 μl

(3) Application: Cloning of β-globin region (9.3 kb) from human genomic DNA

A. Digestion of DNA with restriction enzyme

1) Prepare the following mixture in a tube and incubate at 30℃ for 3 hours.

Human genomic DNA	3 μg
*Bam*H I	30 units
10X Buffer for *Bam*H I	5 μl
Sterilized distilled water	up to 50 μl

2) Collect DNA by ethanol precipitation and dissolve the precipitate in 10 μl sterilized distilled water.

B. Ligation reaction

1) Prepare the reaction mixture shown below and incubate at 16℃ for 30 mins.

A-2) DNA solution	5 μl
*Sau*3A I cassette (X 10 diluted)	2.5 μl
Ligation solution I	15 μl
Ligation solution II	7.5 μl

2) Collect DNA by ethanol precipitation and dissolve the precipitate in 5 μl sterilized distilled water.

C. 1st PCR

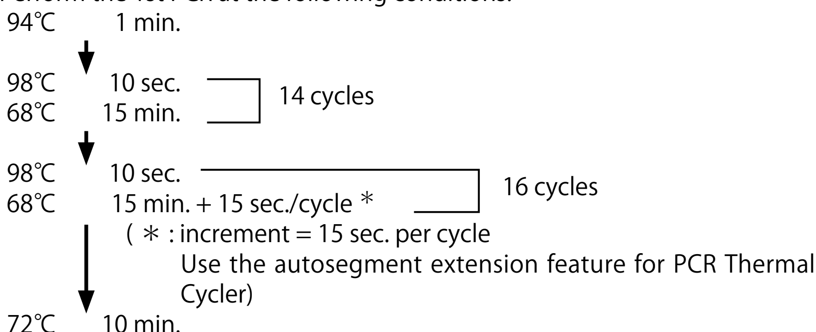
1) Add 1 μl of DNA solution prepared at B-2) to 33.5 μl of sterilized distilled water, and heat 94℃ for 10 minutes.

2) Prepare the following reaction mixture.

C-1) DNA solution	34.5 μl
10X LA Buffer II (Mg^{2+} plus)	5 μl
TaKaRa LA Taq	0.5 μl
dNTP Mixture	8 μl
Primer C1	1 μl
Primer A1	1 μl
total	50 μl

Primer A1 5'-CAGAAAGTGTTTCTGAAAGAGGGATTAGCCCGTTG-3'

3) Perform the 1st PCR at the following conditions.



D. 2nd PCR

1) Dilute some portion of the 1st PCR product and prepare 3 kinds of samples; original, 10 times-diluted, and 100 times-diluted.

2) Prepare the following reaction mixture for each sample respectively for the 2nd PCR.

1st PCR reactant (prepared at D-1)	1 μl
10X LA Buffer II (Mg^{2+} plus)	5 μl
TaKaRa LA Taq	0.5 μl
dNTP Mixture	8 μl
Primer C2	1 μl
Primer A2	1 μl
Sterilized distilled water	33.5 μl
total	50 μl

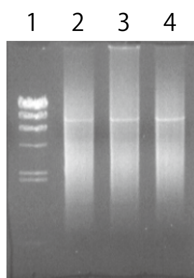
Primer A2 5'-TGCACCTGCTCTGTGATTATGACTATCCCACAGTC-3'

3) Perform the 2nd PCR under the same conditions as in the 1st PCR.

4) Amplified fragment of 9.3 kb was confirmed by the gel electrophoresis. (1% Agarose gel electrophoresis, 5 μl/apply)

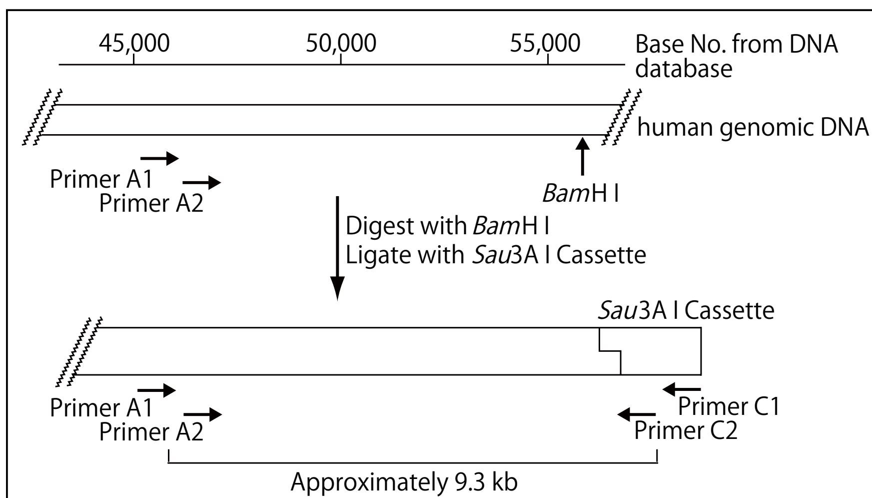
β-globin region (9.3 kb) of human genomic DNA

1 2 3 4



Lane 1 : λ-*Hin*d III digest
2 : 1st. PCR reactant 1 μl
3 : 1st. PCR reactant X 10 diluted 1 μl
4 : 1st. PCR reactant X 100 diluted 1 μl
1% Agarose gel
applied volume : 5 μl

[Principle]



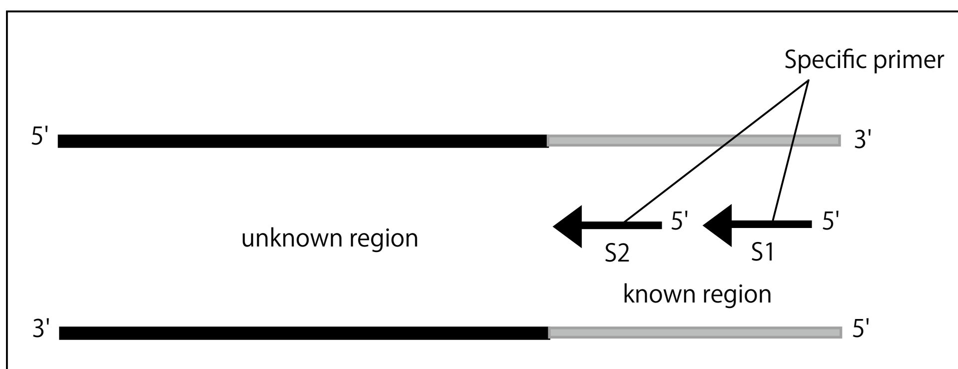
VII. Note

When performing PCR, please note the following points:

1) Mix well the kit components by pipetting or shaking tubes up and down after melting completely at the room temperature -37℃ . It is recommended to avoid mixing vigorously by vortex mixer. Especially when mixing 10X LA PCR Buffer and *TaKaRa LA Taq*, mix carefully and gently to avoid producing bubbles or inactivation of enzyme. Each component should be kept on the ice until used.
2) Mix gently the reaction mixture by pipetting before starting reactions. In this case, never use vortex mixer.
3) Conditions for Annealing and Extension : Determine the optimum annealing temperature experimentally by varying temperatures in 2℃ increments over a range of 45℃ to 68℃ . In three step PCR, the standard extension speed at 72℃ is 1 min./kb. To carry out the combined annealing-extension at 68℃ (two step PCR)＊, the recommendedation time is also 1 min./kb. When temperature are set below 68℃ , longer time will be required.
   ＊ : As *TaKaRa LA Taq* shows sufficient activity at 60℃ to 68℃ , Shuttle PCR (Two step PCR) can be conducted.

VIII. Guidelines for Designing Specific Primer

1. Designing specific primers annealing with known region



Primers should be prepared in the direction to amplify the unknown region. Primer S2 should be designed to anneal with the inner sequence of the annealing sequence of Primer S1. The distance between S1 and S2 does not have substantial influence on results.

Rules :

1) Primer length should be between 20 - 35 mers. (30 - 35 mers is recommended in the amplification of a long DNA fragment (≧ 4 kb).)
2) Primers should have a balanced G/C and A/T content and avoid to be partially rich in G/C or A/T content. Especially 3'-end side of primers should not have high content of A/T.
3) Primers should be designed to avoid taking the secondary structure.
4) As both S1 and S2 specific primers are used in combination with Cassette Primer (C1, C2), especially 3 - 4 mers from 3'end should avoid complementarity to prevent "primer dimer" formation.

2. Designing specific primers based on amino acid sequence of protein

1) When primers are designed on the basis of obtained amino acid sequence, amino acid sequence should be 1st converted into nucleotide sequence. Primer sequence should be selected from the region carrying as less degeneracy as possible. However, in most cases, long primers with more degeneracy work better than short primers with less degeneracy.
2) In order to prepare less degenerate primers, design a primer considering coden usage.
3) Primers should not have mixed nucleotide sequence at 3'end.
4) Using mixed primers requires to decrease annealing temperature, which leads to more nonspecific amplification. When enough sequence information of a known region is already obtained, target DNA can be identified by preparing another primer in more inside areas of a primer.

IX. Sequences of Cassettes and Cassette Primers

***Sau*3A I Cassette**

5' HO GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGA 3'
3'CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTAG OH5'

***Eco*R I Cassette**

5' HO GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGAG3'
3'CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTCTTAA OH 5'

***Hin*d III Cassette**

5' HO GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGA 3'
3'CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTTCGA OH 5'

***Pst* I Cassette**

5' HO GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGACTGCA 3'
3'CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTG OH 5'

***Sal* I Cassette**

5' HO GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGAG3'
3'CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTCAGCT OH 5'

***Xba* I Cassette**

5' HO GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGAT 3'
3'CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTAGATC OH 5'

Cassette Primer C1

5' GTACATATTGTCGTTAGAACGCGTAATACGACTCA 3'

Cassette Primer C2

5' CGTTAGAACGCGTAATACGACTCACTATAGGGAGA 3'

X. Reference

1) Isegawa, Y., Sheng, J., Sokawa, Y., Yamanishi, K., Nakagomi, O. and Shigeharu, U. (1992) *Molecular and Cellular Probes*, **6**, 467-475.
2) Barnes, W. M. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 2216-2220.
3) Cheng, S. et al. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 5695-5699.
4) Cheng, S. et al. (1994) *Nature*, **369**, 684-685.

XI. Related Products

Cassette, *Sau*3A I (Cat. #3873)
Cassette, *Eco*R I (Cat. #3869)
Cassette, *Hin*d III (Cat. #3870)
Cassette, *Pst* I (Cat. #3871)
Cassette, *Sal* I (Cat. #3872)
Cassette, *Xba* I (Cat. #3874)
Cassette, Primer C1 (Cat. #3877)
Cassette, Primer C2 (Cat. #3878)
*Bam*H I (Cat. #1010A/B)
*Eco*R I (Cat. #1040A/B)
*Hin*d III (Cat. #1060A/B)
Pst I (Cat. #1073A/B)
Sal I (Cat. #1080A/B)
Xba I (Cat. #1093A/B)
Bgl II (Cat. #1021A/B)
Fba I (Cat. #1045A/B)
Mfl I (Cat. #1070A/B)
DNA Ligation Kit Ver.2.1 (Cat. #6022)
TaKaRa LA Taq™ (Cat. #RR002A/B)
TaKaRa PCR Thermal Cycler Dice™ Gradient/Standard (Cat. #TP600/TP650)
TaKaRa PCR Thermal Cycler Dice™ mini (Cat. #TP100)

NOTE : This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

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