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The Use of siRNA to Validate Immunofluorescence Studies

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Summary

Cellular immunofluorescence studies can be validated by using either specific small interfering RNA (siRNA) duplexes or expression plasmids that induce the expression of specific siRNAs. The usage of either siRNA tool reduces the expression of the specific protein being studied, thus reducing substantially or abolishing the immunofluorescence detected when using a fluorescent antibody that recognizes the protein.

Key Words: Antibody validation; immunofluoresence; RNA duplexes; RNAi; short hairpin RNA; siRNA.

1. Introduction

RNA interference (RNAi) is a cellular process common to most eukaryotes. The role this process plays in the life of a cell has been extensively investigated *(1–3)*. Functions such as protection against viral infection, regulation of chromatin remodeling, and regulation of gene expression have been determined. RNAi contributions to the etiology of cancer are aggressively being researched. Although the mechanism of RNAi-mediated gene silencing remains to be fully elucidated, the use of RNAi has become a valuable tool for analysis of gene function and target validation. RNAi leads to the inhibition of protein expression by utilizing sequence-specific, dsRNA-mediated degradation of the target messenger RNA (mRNA) *(4)*. In 2001, Tuschl and his colleagues showed that when short RNA duplexes (19–23 bases in length) were introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA was effected without inducing an interferon response *(5)*. These short dsRNA, referred to as small interfering RNA (siRNA), act catalytically at submolar concentrations and can cleave up to 95% of the target mRNA in the cell, substantially reducing expression of the encoded protein. The siRNA-mediated effect has been shown to be relatively stable over time and silencing might be observed through several cell generations *(6)*.

One of the most powerful applications of RNAi is its use in functional genomic screens for gene target identification. Through such programs, it is possible to correlate specific gene targets with specific cellular phenotypes more accurately and quickly than ever experienced in the history of life science. The most critical factor in these screens is the assay that reports the results. Bringing together RNAi and fluorescent antibodies makes possible high content screening (HCS) assays that enable rapid and accurate assessment of cellular phenotypes. With these HCS assays, the specific genes responsible for a cellular phenotype can be rapidly identified in a cell-based format in which proteins and protein modifications are being monitored. Research programs using this technology have been responsible for identifying gene targets that drug discovery programs the world over are utilizing as targets in inhibitory compound screens. RNAi can be used to validate the specificity of the antibodies being used in immunofluorescent HCS assays.

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

It is important when evaluating cellular immunofluorescence studies to confirm the specificity of an antibody's reaction with its respective antigen and assess the presence of any cross-reactivity. In the past, specificity has been evaluated in cellular immunofluorescence studies by using immunizing peptides as inhibitory controls. These peptide reagents block the action of the antibody, but they do not validate the antibody as being specific for its respective protein antigen. siRNA duplexes and expression plasmids that induce the expression of siRNAs specific for a single gene target can be effectively used to validate immunofluorescence studies. The use of siRNA reagents to knock down the expression of a specific protein antigen can then be observed by a lack of fluorescence when using a specific antibody in a cellular immunofluorescence study. This chapter describes the use of siRNA duplexes and expression plasmids that encode specific siRNAs in cellular immunofluorescence studies to validate an antibody's immunoreactivity to its specific protein antigen.

2. Materials

2.1. Plating of Cells for Transfection

1. Human HeLa cells or another adherent cell line model.
2. Sterile six-or 96-well Costar® tissue culture plates (Corning, Corning NY).
3. Round glass cover slips for six-well plate studies (Fisher Scientific Int., Pittsburgh PA).
4. Dulbecco's minimal essential medium (DMEM) culture media (Mediatech Inc., Herndon VA).
5. Tissue culture sterile fetal bovine serum (FBS) and penicillin-streptomycin (HyClone, Logan UT).
6. Cell culture incubator with 5% CO_2.

2.2. siRNA Duplexes Method

1. Specific siRNA duplexes (5 nmoles SMARTpool® from Dharmacon, Boulder, CO or Upstate Group LCC; Charlottesville, VA).
2. Nonspecific control siRNA duplexes (1 nmole from Dharmacon or Upstate).
3. 5X siRNA buffer: 100 m*M* KCl, 1 m*M* $MgCl_2$, 30 m*M* HEPES, pH 7.5; or available from Upstate.
4. Sterile RNAase-free water.
5. Transfection reagent of choice, such as siIMPORTER™ (do not freeze) (Upstate).
6. 1.6-mL microcentrifuge tubes.

2.3. siRNA Expression Plasmid Method

1. siRNA plasmid DNA preferably in pKD™ vector (Upstate).
2. Transfection reagent of choice, such as FuGene6 transfection reagent (Roche Diagnostics, Alameda CA).

2.4. Cellular Immunofluorescence Method

1. Phosphate-buffered saline (PBS): 150 m*M* NaCl, 100 m*M* phosphate buffer, pH 7.2.
2. Appropriate cell fixative for retaining protein antigencity (either 95% ethanol/5% acetic acid, or 50% methanol 50% ethanol, or 1% paraformaldeyde in PBS (Sigma, St. Louis, MO).
3. Triton X-100 (Sigma).
4. Primary antibody for protein of interest, preferably directly conjugated to a red fluorophore (e.g., AlexaFluor 555®; Molecular Probes/Invitrogen, Eugene OR).
5. An appropriate secondary antibody conjugated to a fluorophore if the primary antibody is not directly conjugated to a fluorophore (Molecular Probes).
6. ProLong Gold® slide mounting medium for immunofluorescence (Molecular Probes). This reagent contains DAPI to stain the nuclei blue.
7. Fluorescent microscope or HCS instrument with appropriate filters.

3. Methods

3.1. Plating of Cells for Transfection

1. Plate HeLa cells or a desired cell line model in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin in either a 96-well tissue culture plate (2K cells/well) or onto glass cover slips in a 6-well tissue culture plate (80K cells/well) (*see* **Note 1**).

2. Incubate cells for 24 h at 37°C and 5% CO_2 to achieve 70–90% confluency before transfection with either siRNA duplexes or expression plasmids (*see* **Note 2**).

3.2. The Usage of Specific siRNAs Duplexes

The selection of functional siRNAs is one of the major issues confronting the RNAi application. This selection requires sophisticated selection criteria to identify highly active siRNAs. It is recommended that scientists wishing to use siRNA as a validation tool strongly consider using commercial providers for these reagents. We have been exclusively using Dharmacon's pooled siRNA reagents. Dharmacon has developed two programs (SMARTselection and SMARTpooling) that have been successful at designing effective siRNAs. SMARTselection uses an algorithm consisting of 33 criteria and parameters that effectively eliminate nonfunctional siRNAs. SMARTpooling uses a sophisticated algorithm to combine four or more SMARTselected siRNA duplexes in a single pool. Each Dharmacon siRNA pool reduces mRNA levels by at least 70% and many will reduce mRNA levels by 95%. We routinely use the siIMPORTER™ reagent to transfect siRNA duplexes into mammalian cells (*see* **Note 3**). The siIMPORTER transfection reagent is a cationic lipid formulation that has been developed for efficient transfection of siRNA duplexes into mammalian cells.

It is important to determine the optimal time-point for knockdown of the target protein's expression after transfection of cells with siRNA reagents. The half-life of cellular proteins can vary considerably. In general, the mRNA levels of target proteins are usually significantly reduced by 24–48 h post-transfection. The best time period for showing the knockdown of most protein targets; however, generally occurs between 72 and 96 h post-transfection.

3.2.1. Transfection of Cells Using siRNA Duplexes

1. Dilute the 5X siRNA Buffer to 1X by mixing four volumes of sterile RNase-free water with one volume of 5X siRNA Buffer.
2. Each Dharmacon SMARTpool® contains 5 nmoles of material and each nonspecific control contains 1 nmole of material. The siRNA should be resuspended using 250 µL of 1X siRNA Buffer for a recommended concentration of 20 µ*M* (20 pmol/µL). Final concentration ranges from 1 to 200 n*M* should be used in initial experiments so that the optimal concentration for the knock down of the protein target can be determined for the assay.
3. The siRNA nonspecific control pool should be resuspended using 50 µL of 1X siRNA Buffer for a recommended concentration of 20 µ*M* (20 pmol/µL). We recommend using a negative control siRNA in every set of transfection studies at the same concentration as the experimental siRNA (*see* **Note 4**). We also recommend including untransfected or mock transfected cells as an additional negative control in siRNA studies.
4. For lipid complex formation and subsequent transfection, we recommend following the instructions provided by the transfection reagent manufacturer and taking measures to test and optimize the conditions best suited for the cell line of choice. We recommend using cell densities at approx 70–90% confluent (approx 1×10^5 cells/mL density for 96-well plates) at the time of transfection. The optimal cell number necessary to achieve this amount of confluence will vary with the growth characteristics of the cells.
5. For transfection of cells with siIMPORTER, use a microcentrifuge tube to first mix the siIMPORTER reagent with serum-free medium (Tube 1). For 96-well experiments, mix 0.5 µL of siIMPORTER with 2.5 µL of serum-free medium. For six-well plate experiments, mix 5.0 µL of siIMPORTER with 25 µL of serum-free medium.
6. In a second microcentrifuge tube (Tube 2), mix siRNA diluent and serum-free medium together and then add either the specific siRNA pool or the nonspecific control siRNA. The siRNA diluent promotes complex formation between siRNA and siIMPORTER. For 96-well experiments, mix 2.0 µL of siRNA diluent with 1 µL of serum-free medium followed by the addition of the siRNA. Adding 0.5 µL of a 20 µ*M* siRNA preparation will give a final concentration in the reaction mixture of 100 n*M*. For six-well plate experiments, mix 25.0 µL of siRNA diluent with 10 µL of serum-free medium. To this mixture, add 5.0 µL of the 20 µ*M* siRNA preparation to achieve a 100 n*M* final concentration. Mix gently by pipeting. Do not vortex.

7. Add the siRNA solution prepared in **step 6** (Tube 2) to the diluted siIMPORTER solution prepared in **step 5** (Tube 1). Incubate this mixture for 5 min to allow siRNA/lipid complexes to form. Do not incubate this mixture for longer than 30 min before use or transfection efficiency might be diminished.
8. For 96-well experiments, add 7.0 µL of the siRNA/siIMPORTER mixture with 93 µL of media. For 6-well plate experiments, add 70 µL of the siRNA/siIMPORTER mixture with 930 µL of media. With some cell types, higher transfection efficiencies are seen if serum is not present during the first 4 h of incubation. In these experiments, a small aliquot of media containing 20% FBS can be added after 4 h of incubation.
9. For immunofluorescence studies, incubate the cells for 72–96 h at 37°C and 5% CO_2 to detect siRNA-induced knockdown of protein targets.

3.2. The Usage of Specific siRNA Expression Plasmids

3.2.1. siRNA Oligonucleotide Design and Cloning Into pKD

We routinely use an Upstate-developed expression vector, pKD. This vector was designed to receive double stranded DNA oligonucleotides so that when the resulting plasmid is transfected into mammalian tissue culture cells, the cloned sequence gets transcribed and processed into a functional siRNA. A schematic of the pKD plasmid is shown in **Fig. 1**. A brief description of the design and usage of this expression vector is as follows: a double-stranded, annealed DNA oligonucleotide is generated that corresponds to the target gene mRNA sequence such that the target gene sense sequence is represented 5′ of its antisense and is separated by a 8 bp "loop" region. The DNA oligonucleotides are designed with the first 22 nucleotides being the sequence used in the siRNA for the target gene using a highly advanced search algorithm to identify nonredundant sequences in the genome. This oligo is then cloned into the pKD expression vector, which uses the human HI, RNA polymerase III-based promoter to express the cloned sequence. The RNA transcript produced by the pKD vector's HI promoter is terminated by the dT5 sequence immediately 3′ of the cloned oligos. The transcript is then able to fold onto itself as the sense and antisense regions are able to basepair. The eight nucleotide "loop" region allows for the short hairpin RNA to form. Cellular ribonucleases process the short hairpin RNA into a siRNA, which is fully functional for RNAi-mediated degradation of a particular mRNA target.

3.2.2. Transfection Complex

1. Plate cells for 24 h before transfection as described in **Subheading 3.1.**
2. In a sterile microcentrifuge tube, combine the following in the prescribed order, as the order of addition of components to the complex mixture is important (*see* **Note 5**):

	96-well	*6-well*
Serum-free DMEM	9.425 µL	94.3 µL
FuGene6 transfection reagent	0.075 µL	0.75 µL
siRNA plasmid DNA (0.1 mg/mL)	0.5 µL	5 µL
Total	10 µL	100 µL

3. Mix tube contents by gently tapping (three to four times).
4. Incubate at room temperature for a minimum of 15 min but not for more than 45 min as this might affect transfection efficiency.

3.2.3. Plasmid Transfection

1. Do not remove the old media from the cell culture.
2. Slowly add the transfection complex to each tissue culture well while gently swirling the plate. Use 10 µL of the transfection complex for each well of a 96-well plate and 100 µL for each well of a 6-well plate containing a glass cover slip.
3. Incubate the cells at 37°C and 5% CO_2 for 72–96 h before using cells for immunofluorescence studies.

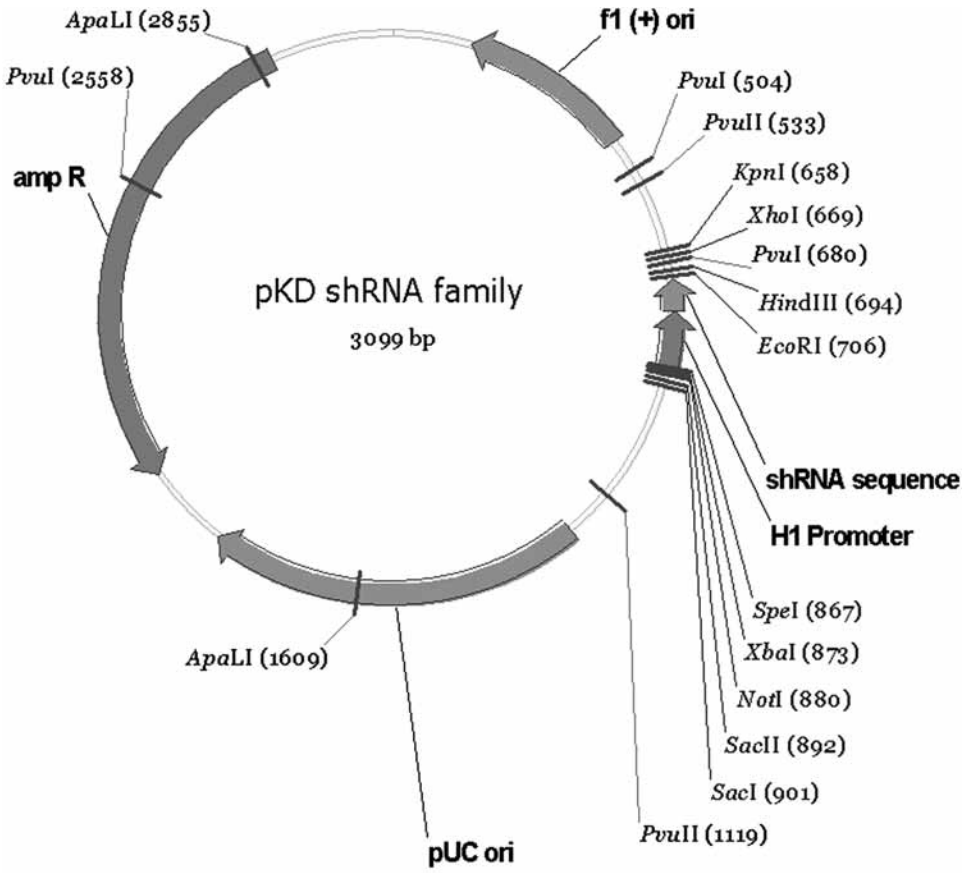

Fig. 1. A schematic drawing of the pKD expression plasmid used to induce the expression of specific short hairpin RNAs in mammalian cells. Cellular ribonucleases process the short hairpin RNAs into functional, siRNAs. (Please *see* the companion CD for the color version of this figure.)

3.3. Immunofluorescence of siRNA-Treated Cells

1. Carefully aspirate the culture media and rinse cells carefully with PBS.
2. Aspirate the PBS and then carefully add fixative. The suitable fixative must be determined empirically for each cell system studied. Three of the most commonly used fixatives are: (1) 95% ethanol/5% acetic acid; (2) 50% methanol/50% ethanol, and (3) 3.7% formaldehyde in PBS. We routinely fix cells at room temperature for 20 min using 3.7% formaldehyde.
3. Immediately wash the cells twice for 5 min with PBS. Do not shake.
4. Aspirate the PBS and add a cellular permeabilization agent. We routinely use 0.5% Triton X-100 (diluted in PBS) for 2 min. Permeabilization conditions and the reagents used, however, it might need to be modified for each cell type.
5. Optional blocking step: with some antibodies an optional blocking step might be needed to reduce background cellular fluorescence. In those instances, cover cells with 8% BSA in PBS and incubate for 1 h at room temperature. Perform the incubation in a sealed humidity chamber to prevent air drying of the cells. Wash cells twice for 5 min with PBS afterwards if this step is necessary.
6. Gently remove excess PBS and cover cells with the primary antibody of choice diluted in PBS. The antibody can be diluted in 1% BSA if background fluorescence is a concern. Incubate for 1 h at room temperature in a humidity chamber in the dark. Ideally, use a primary antibody directly conjugated to a red fluorophore (e.g., AlexaFluor 555). The optimal concentration of the primary antibody will need to be empirically determined (*see* **Note 6**).

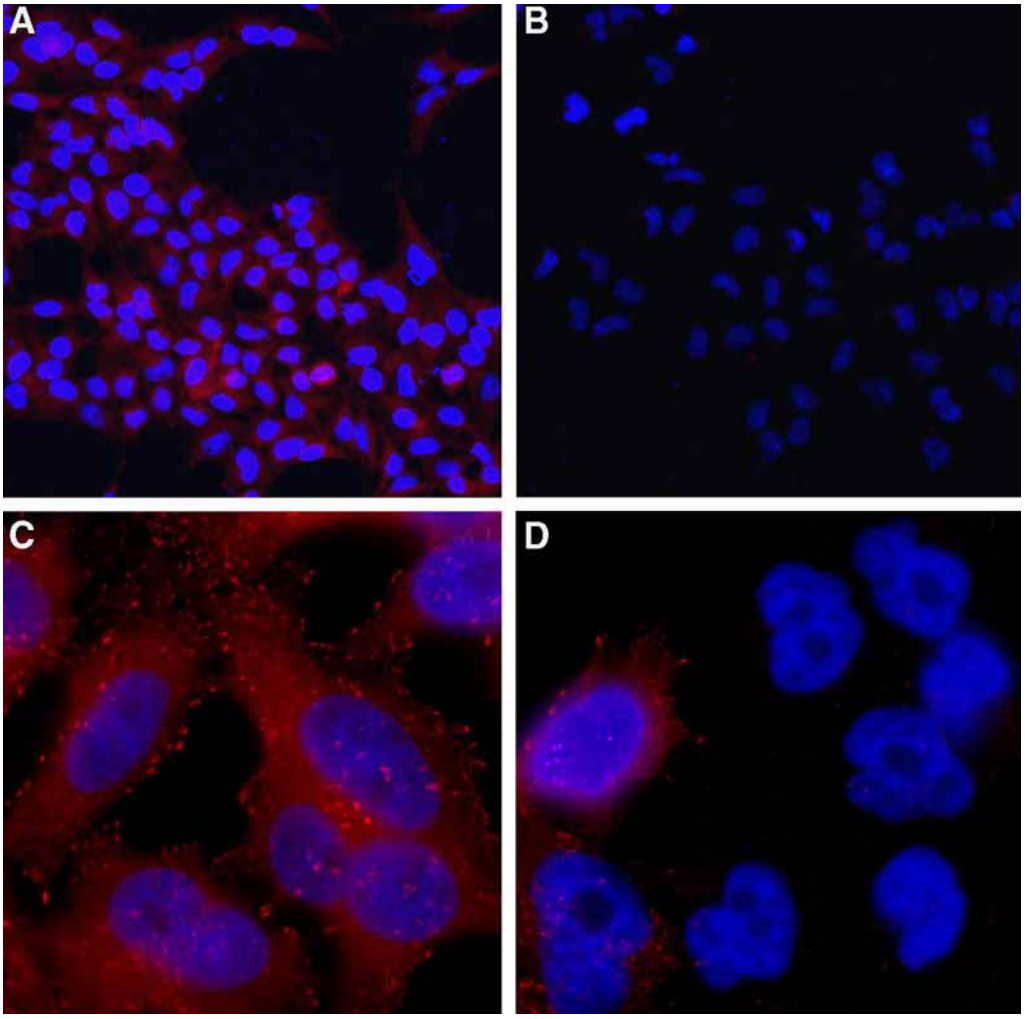

Fig. 2. Use of a pKD expression plasmid to block the expression of focal adhesion kinase (FAK) in Human HeLa cells. (**A,C**) HeLa cells were transfected with pKD-NegCon-v1, a nonspecific pKD expression plasmid (Upstate) and incubated for 96 h at 37°C. The cellular expression of FAK was then evaluated by immunofluorescence using an AlexaFluor 555®-conjugated monoclonal FAK antibody (2 µg/mL; Upstate). Note the fine particulate membrane staining of FAK. (**B,D**) HeLa cells were transfected with pKD-Fak-v1, a short hairpin RNA expression plasmid specific for Fak (Upstate) and incubated for 96 h at 37°C. The cellular expression of FAK was then evaluated by immunofluorescence as in **A** and **C**. The exposure time for each image was equivalent. The cellular level of FAK protein is dramatically reduced and only occasional cells (likely untransfected) show FAK staining similar to that seen in control reactions. Cell nuclei were stained blue using DAP. Images **A** and **B** are at ×20 magnification; images **C** and **D** are at ×100 magnification.

7. Wash the cells three times for 5 min with PBS. If a fluorophore-conjugated primary antibody was used, go directly to **step 10**; if a secondary antibody conjugated to a fluorophore is needed to visualize the target protein, go to **step 8**.
8. If a fluorophore-conjugated primary antibody is not available, gently remove excess PBS and incubate cells with a fluorescent-conjugated secondary antibody of choice in PBS for 1 h at room temperature in the dark. Add 1% BSA as a blocking reagent to the antibody preparation if necessary. The optimal concentration of the fluorescent-conjugated antibody will need to be empirically determined. Perform the incubation in a darkened, humidity chamber.

9. Wash the cells three times for 5 min with PBS in the dark.
10. Mount the cover slip onto a slide using Prolong Gold® and examine the specimen under a fluorescent microscope or in a high content analysis instrument. If you are studying a nuclear protein, then use a similar mounting medium from Molecular Probes that does not contain DAPI.

Figure 2 shows RNAi immunofluorescence experiments using a focal adhesion kinase pKD expression plasmid to knockdown expression of focal adhesion kinase (**Fig. 2B,D**). A nonspecific pKD expression plasmid is used as a control (**Fig. 2A,C**).

4. Notes

1. Avoid using high passage number cells and cell cultures less than 40% confluent for your siRNA experiments. These cell cultures can give inconsistent results.
2. The transfection protocol described was optimized for use with HeLa cells. For other cell lines, it is highly recommended that you optimize your transfection conditions because plating density and cell type greatly influence transfection efficiency. In addition, other cell lines might require a different transfection reagent.
3. If you observe low transfection efficiency, optimize the siIMPORTER/siRNA ratio by varying the amount of siIMPORTER and keeping a constant amount of siRNA.
4. RNA oligos are susceptible to degradation by Rnases, which are present almost everywhere. For this reason, they should be handled and stored using RNase-free conditions and solutions. Gloves should always be worn during handling and solutions should be treated to inhibit or destroy ribonucleases.
5. A master mix of the serum-free DMEM and FuGene6 can be made based on the number of samples to be tested and then aliquoted into tubes prior to DNA addition. The FuGene6 must be added directly into the serum free DMEM media. Do not let FuGene6 touch any plastic other than the pipet tip.
6. We recommend that the specificity of the primary antibody for the protein target is confirmed using a secondary method such as western immunoblotting. It is important to show that the primary antibody recognizes a single protein band in an immunoblot to assert specificity.

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