Development of an AlphaLISA assay to measure and screen inhibitors of the p53-MDM2 interaction.

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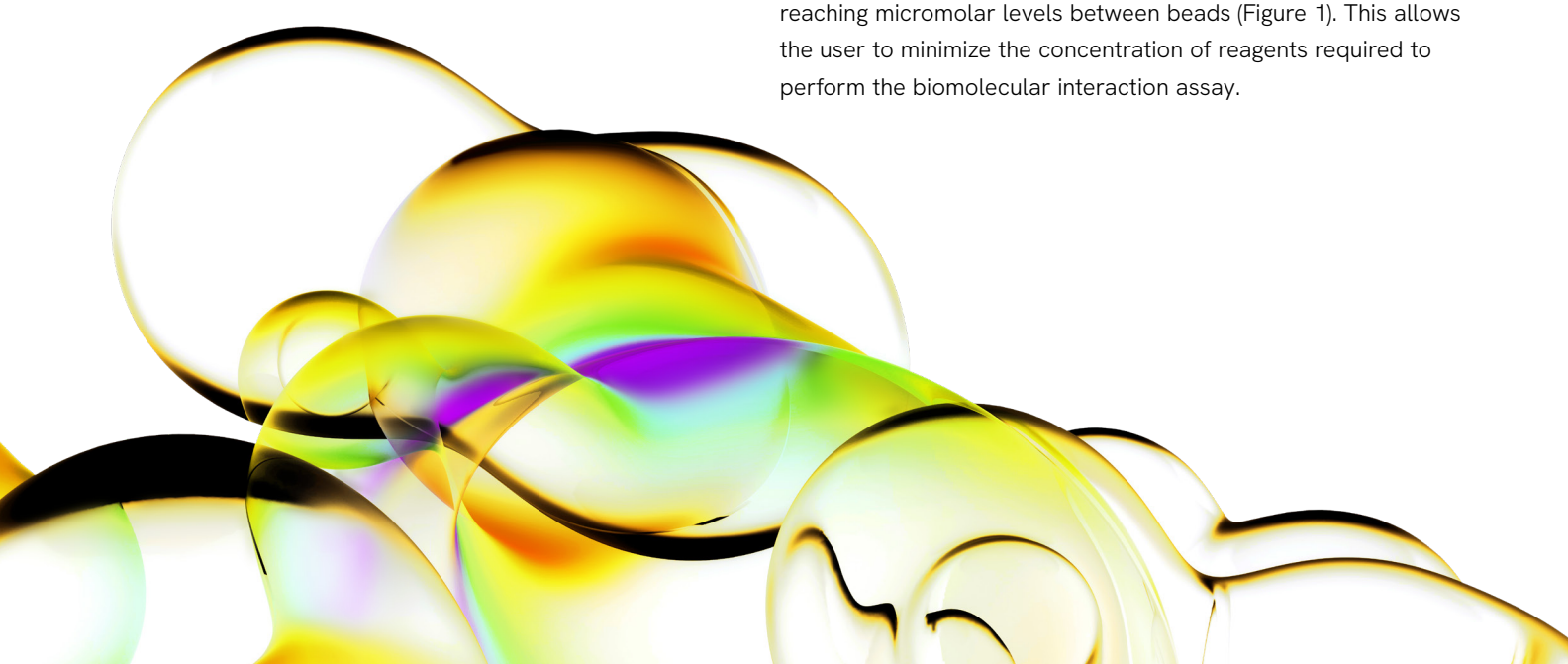
Introduction

Biomolecular interactions, or binding events between biomolecules, are important components of biological processes such as transcription, translation, and post-translational modifications used for cell signaling. A number of these binding events have been targeted for the development of novel therapeutic drugs. In fact, there are many drugs on the market specifically targeting protein-protein interactions. There are a variety of assay formats to measure binding events; however, efficient, highly sensitive assays that can be used to study a large range of binding affinities are extremely important for validating therapeutic drugs. One of the many applications of AlphaLISA™ technology is the ability to investigate protein-protein interactions.

AlphaLISA is a bead-based homogeneous technology that can be used to study a large range of biomolecular interactions in a microplate format. Alpha assays require two types of beads: Donor beads and Acceptor beads. Typically, each bead is conjugated to a protein or antibody used to capture one of the targets in the biomolecular interaction assay. When the two biomolecules interact, the Donor bead is brought into proximity of the Acceptor bead and excitation of the Donor bead results in a luminescent signal from the Acceptor bead. AlphaLISA can detect a broad range of affinities with dissociation constants (K_D) ranging from picomolar to low millimolar. Since each bead has multiple binding sites, when one bead-biomolecule binding event takes place, this facilitates the other biomolecule binding to the bead. Low nanomolar concentrations of binding partners can generate high local concentrations of protein complexes reaching micromolar levels between beads (Figure 1). This allows the user to minimize the concentration of reagents required to perform the biomolecular interaction assay.



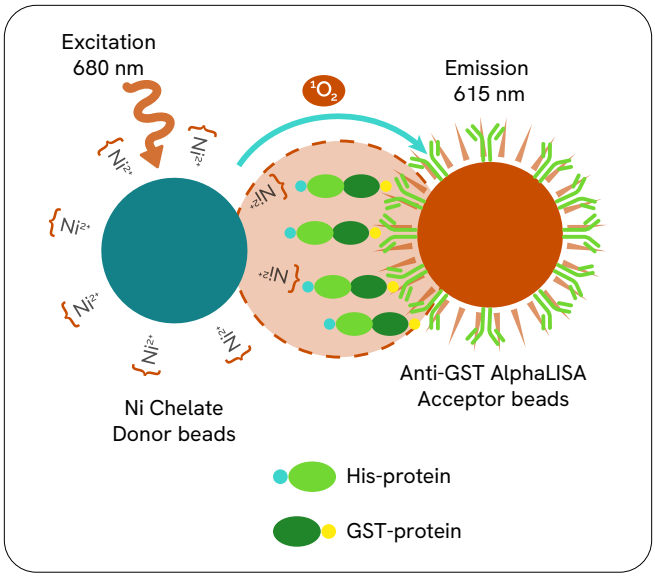


Figure 1: Avidity of AlphaLISA beads increases local concentrations of bound biomolecules.

p53 is a transcription factor and tumor suppressor protein that is activated in response to cellular stress. MDM2 was identified as a negative regulator that binds to p53 and tags it for ubiquitination and subsequent degradation.[1] Prior research has shown that these proteins bind with a dissociation constant (K_D) in the range of 300 nM.[2] This protein-protein interaction has been an excellent target for therapeutic drugs, and therefore, we have chosen it as a model system for developing an AlphaLISA assay to screen for inhibitors of the interaction. Figure 2 shows a representative schematic of one possible set-up to look at the p53-MDM2 interaction using AlphaLISA. In this technical note, we will not only show how to develop an assay to screen for inhibitors, but also show how to measure a dissociation constant for this moderate binding protein-protein interaction using AlphaLISA.

Materials and methods

Instrumentation

All AlphaLISA measurements were performed on the Revvity 2105 EnVision™ multimode plate reader using standard Alpha settings.

Reagents

- GST-tagged MDM2 (Boston Biochem, #E3-202)
- His-p53 (Boston Biochem, #SP-405)
- Anti-GST AlphaLISA Acceptor Beads (Revvity, #AL110),
- Anti-6xHis AlphaLISA Acceptor Beads (Revvity, #AL128)
- Ni chelate AlphaLISA Acceptor Beads (Revvity, #AL108)
- GSH AlphaLISA Acceptor Beads (Revvity, #AL109)
- Ni chelate Alpha Donor Beads (Revvity, #AS101)
- GSH Alpha Donor Beads (Revvity, #6765300)
- Nutlin-3a (SelleckChem, #S8059)
- Untagged p53 (Boston Biochem, #SP-454)
- AlphaPlate™-384, light gray (Revvity, #6005350)

AlphaLISA assay protocols

Protein-protein interaction assay: A cross-titration of GST-tagged MDM2 (10 µL) with His-p53 (10 µL) was incubated for one hour. Then 10 µL of either anti-GST, anti-His, Ni chelate or GSH AlphaLISA Acceptor beads were added (20 µg/mL final) and incubated for one hour. Finally, 10 µL of either Ni chelate or GSH Donor beads were added and incubated for one hour before measuring AlphaLISA signal.

Competition assay: 5 µL of GST-MDM2 (1 nM final) was incubated with 10 µL of various concentrations of Nutlin-3a and 5 µL His-p53 (1 nM final) for one hour. Then 10 µL of GSH AlphaLISA Acceptor beads (20 µg/mL final) were added for one hour. Finally, 10 µL Ni chelate Donor beads (20 µg/mL final) were added and incubated for one hour before measuring AlphaLISA signal.

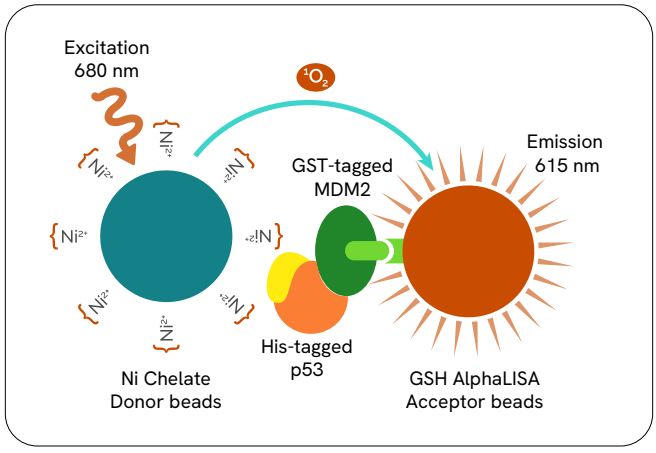


Figure 2: Detection of p53-MDM2 interaction using AlphaLISA.

K_D determination: Competition format was repeated (same volumes, incubation times, and bead concentrations) with a titration of untagged p53 using 30 nM (final) His-p53 and 3 nM (final) GST-MDM2 and the K_D estimated to be equivalent to the IC_{50}.

Z′ calculation: Assay set-up was the same as the competition format for protein and bead addition. One dose of Nutlin-3a (50 µM) was selected as the positive control and assay buffer alone represents the negative control.

Data analysis

The data were analyzed using GraphPad Prism® software. Binding curves were generated using nonlinear regression, using a four-parameter logistic equation (sigmoidal dose-response curve with variable slope). The inhibition curves were generated using log (inhibitor) vs. response - variable slope (four parameters).

For Z′ determination, we used the equation:
Z′ = 1-(3*(standard deviation (high signal) + standard deviation (low signal)) / (average (high signal) - average (low signal)).

Results

Optimizing protein-protein interaction assay

The first step in developing an AlphaLISA assay for protein-protein interactions is to identify the options for capturing the proteins on the beads depending on the protein tag. For these experiments, we used a GST-tagged MDM2 and a His-tagged p53. For these two tags, there are four different bead combinations possible: GSH Donor with either anti-His or Ni chelate AlphaLISA Acceptor beads or Ni chelate Donor beads with either GSH or anti-GST AlphaLISA Acceptor beads.

We performed a cross-titration of the proteins in the range of 0.3 to 30 nM (final concentration) and tested each of the four different bead combinations. As shown in Figure 3, many of the combinations gave excellent signal to background and we chose to move forward with Ni chelate Donor beads with GSH AlphaLISA Acceptor beads. We found that 10 nM MDM2 and 30 nM p53 showed the best signal to background for this bead pair.

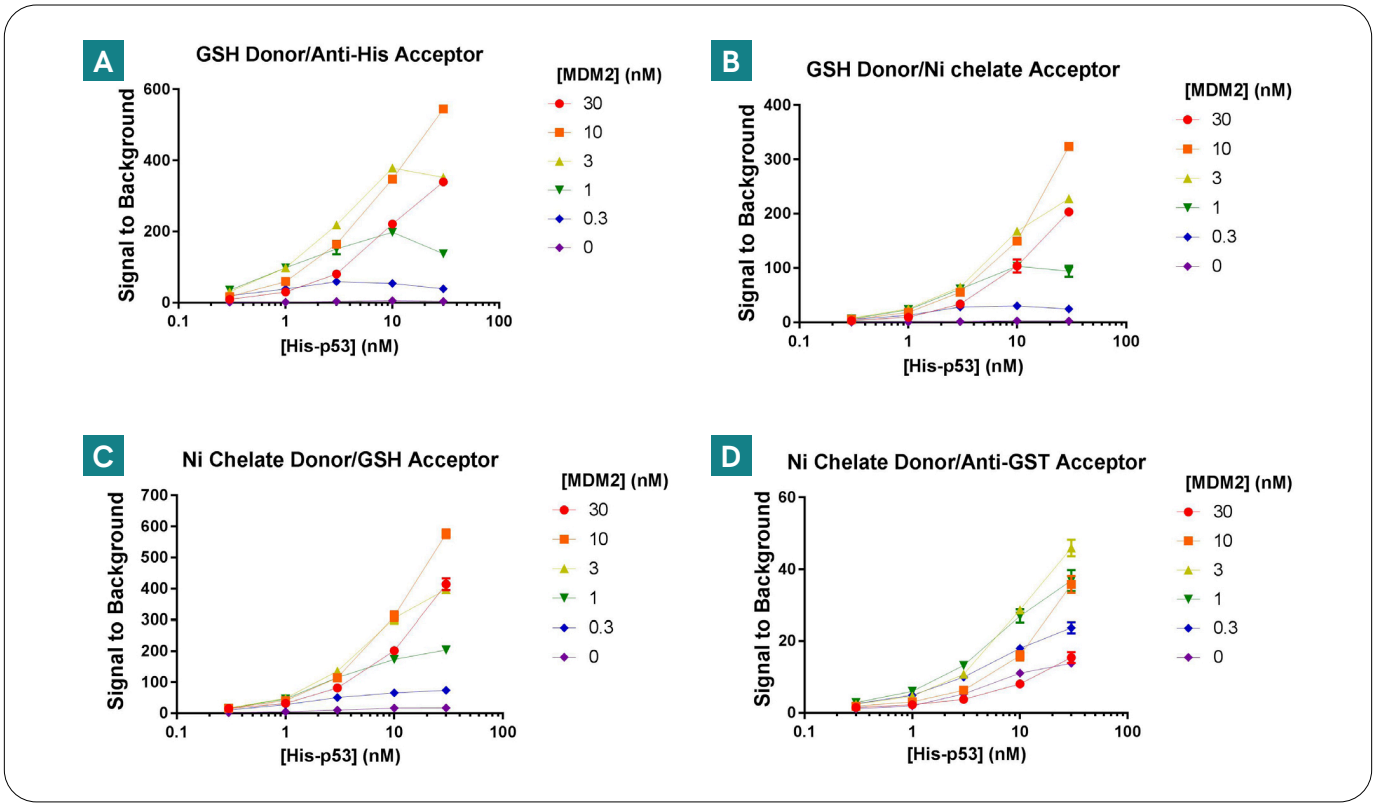


Figure 3: Protein-protein cross-titration and bead pair investigation.

Specificity of interaction and utility for screening inhibitors

We observed that a 1:1 ratio of proteins (1 nM final concentration of each protein) provides sufficient signal to background. Therefore, to conserve protein, we used this ratio for further studies. To show the utility of this assay to screen inhibitors and to verify the specificity of the signal, we performed a competition assay with a known compound inhibitor of the interaction, Nutlin-3a. As shown in Figure 4, we see a competition curve indicative of disrupting this interaction. The IC_{50} of Nutlin-3a for this assay at a 1:1 ratio 1 nM final concentration of each protein was 1.3 µM.

K_D Determination for protein-protein interactions with AlphaLISA

In addition to screening inhibitors, AlphaLISA can also be used to measure dissociation constants (K_D) for protein-protein interactions. In limited situations, saturation curve assays can be performed to determine K_D for the protein-protein interaction in Alpha format. Saturation curves can be used to determine the K_D only if the K_D for the protein-protein interaction is low enough such that all protein concentrations used to derive the K_D fall below the bead binding capacities. However, the K_D value for moderate binding interactions is sometimes higher than the capacity of the AlphaLISA beads. Therefore, instead of a saturation curve, a K_D can be determined using a competition assay where an untagged version of one biomolecule displaces the tagged version leading to a decrease in signal. The K_D value can then be calculated from the IC_{50} value based on a method introduced by Cheng and Prusoff[3] shown in Figure 5.

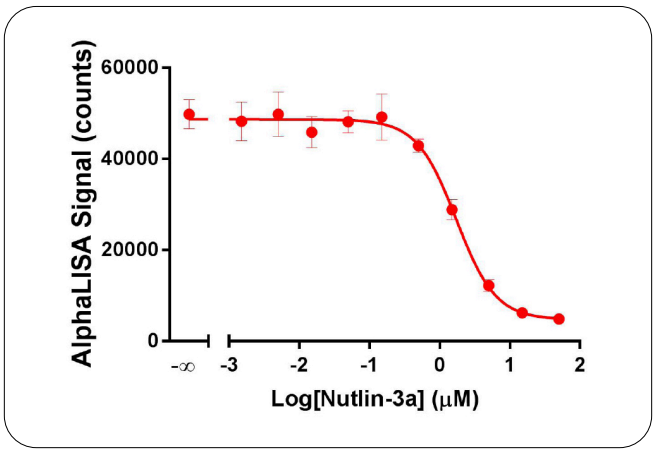


Figure 4: Specificity of interaction using a titration of known inhibitor.

$$[T] \overset{10X}{<<} [L^*] \overset{10X}{<<} K_{D\,(L^*T)}$$

$$K_D = \frac{IC_{50}}{1 + \frac{[L^*]}{K_{D\,(L^*T)}}}$$

$$K_D = \frac{IC_{50}}{1 + \frac{[L^*]}{K_{D\,(L^*T)}}} \to 0$$

$$K_D = IC_{50}$$

Figure 5: Cheng-Prusoff Equation. T is the target and L is the ligand. K_D is the dissociation constant for the binding of the target (T) and ligand (L).

The equation in Figure 5 is written from the point-of-view of a receptor-radioligand binding assay. In an Alpha protein-protein interaction assay, the "target" becomes one of the tagged proteins being caught to the bead, and the "ligand" becomes the second tagged protein binding to the other bead. If the K_D is significantly higher than the concentration of either protein, the value for $[L]/K_D$ in the Cheng and Prusoff equation approaches zero. The full equation then becomes $K_D = IC_{50}/ (1+0)$, and the K_D approximates the IC_{50} in the competition binding assay.

To derive a K_D in this type of assay, a few criteria need to be met:

1. The K_D needs to be at least 10X higher than the concentration of either tagged protein used in the optimized assay. These protein concentrations should be chosen from the first cross-titration experiment, keeping in mind that it is not necessary to pick the concentrations that give the highest signal-to-background. So long as the assay window is acceptable, lower concentrations may be chosen.

2. The concentrations of each tagged protein used in the assay need to be below the binding capacity of their respective bead.

3. The concentration of one labeled protein ("target") should be at least 10X below the concentration of the other labeled protein ("tracer"). If you are using tagged Protein Y, untagged Protein Y, and tagged Protein X in the competition assay, tagged Protein X is the target and tagged Protein Y is the tracer.

Essentially, a displacement assay will be used. However, it will be important to choose concentrations of the untagged protein that are centered around the expected K_D, to derive an accurate IC_{50}. Ideally, a range of concentrations of untagged protein ranging from 0.01X to 100X the K_D would be used. If the expected K_D is not known, it may be necessary to perform two experiments – one using a very broad range of untagged protein concentrations, then once this data has been evaluated, a second experiment can be set up using a narrower concentration of untagged protein. Additionally, it is advisable to test the assay with two or more different concentrations of tagged proteins (that still adhere to the two criteria above) to see if the IC_{50} changes significantly, depending on the tagged protein concentration. If the IC_{50} does vary dramatically between the two concentrations tested, it is likely the lower concentration is more accurate – the assay may need to be re-run with still lower concentrations of tagged protein. An excellent example of the use of competition binding curves to determine affinity in an Alpha assay format is in the paper by Lazar et. al where the IC_{50} of the interaction between Fc variant antibodies and the Fc gamma receptor measured with Alpha was determined to be in close agreement with SPR data.[4]

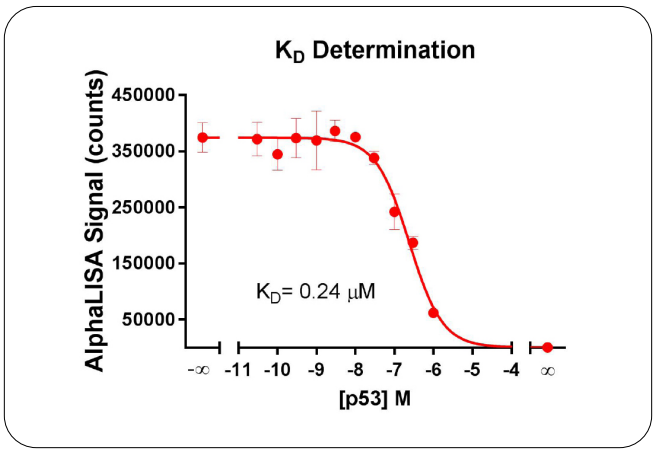


Figure 6: K_D determination using 30 nM His-p53 and 3 nM GST-MDM2.

We followed this method to measure the K_D for the p53-MDM2 interaction using our AlphaLISA assay. As the published value for the K_D was 300 nM we chose 30 nM His-p53 and 3 nM GST-MDM2 for the competition assay. This was also chosen so that the concentrations of both proteins are below the binding capacity of their respective bead (~300 nM-1 µM). An untagged version of p53 was titrated to compete with the interaction. As shown in Figure 6, we can easily determine an accurate K_D for the p53-MDM2 interaction that is consistent with literature values of 300 nM. The data here show that AlphaLISA is amenable to detecting moderate affinity interactions and can accurately measure K_D values using the competition binding method.

Assay robustness

Finally, the Z′ calculating the robustness of the assay for screening purposes was measured using 1 nM of each protein with or without the addition of 50 µM Nutlin-3a in a 384-well format. Figure 7 shows that this assay has a high Z′ (0.70) indicating its suitability for high throughput screening for inhibitors of this interaction. The result also highlights the quality of data obtainable through an AlphaLISA measurement of a moderate affinity protein-protein interaction.

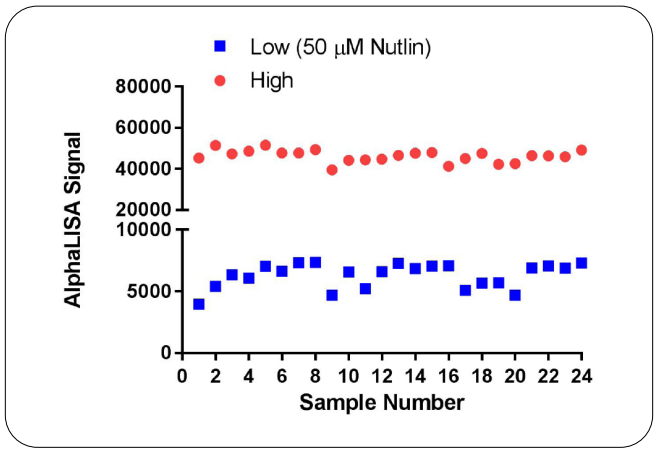


Figure 7: Robustness of assay (Z′).

Summary

In this technical note, we demonstrate how versatile AlphaLISA technology is for setting up a binding assay for a protein-protein interaction with a moderate binding affinity. We also show how easy it is to use AlphaLISA assays to screen for inhibitors of these interactions and demonstrate a high Z′ can be achieved with minimal assay development. We explain how a carefully designed assay can be used to measure dissociation constants (K_D) for protein-protein interactions through a competition binding format. The alternate K_D determination utilizing saturation curves may be more suitable to other protein-protein interactions based on the affinity of the interaction pair, the protein tags, and Alpha bead pairs selected. The versatility of AlphaLISA allows the freedom to utilize the same assay technology whether screening inhibitors with a large range of affinities or looking at a variety of binding partners for a single biomolecule.

References

1. Haupt et al. MDM2 promotes the rapid degradation of p53. Nature (1997); 387: 296-299.

2. Dawson et al. The N-terminal domain of p53 is natively unfolded. J. Mol. Biol. (2003); 332: 1131-1141.

3. Cheng Y, Prusoff WH. Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol. (1973); 22 (23): 3099–108.

4. Lazar et. al. Engineered antibody Fc variants with enhanced effector function. Proc Natl Acad Sci (2006); 103: 4005-10.

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