APPLICATION NOTE

IMMUNOCORE nicoya

Label-free Characterization of ImmTAC® Bispecific Molecules for T-Cell Redirecting Immunotherapy using Alto™

Summary

Bispecific proteins offer a promising new modality for the treatment of cancer and have subsequently gained attention in recent years as the next generation of cancer immunotherapy. In this application note, we demonstrate Alto's applicability to the development of T-cell redirecting bispecific proteins as therapeutic agents for cancer. Alto is used to accurately measure affinities spanning 5 orders of magnitude from 8 bispecific ImmTAC® molecules developed by Immunocore, and provide affinity and off-rate ranking that is equivalent to that obtained with conventional SPR.

Introduction

Bispecific proteins are artificial molecules with two different binding sites designed to target two different epitopes either on the same antigen or two different antigens. Due to this ability to exert two different functions, bispecific proteins are thought to have superior therapeutic effects compared to other antibody-based therapies, and thus have been extensively studied for the treatment of a wide range of diseases.

While therapeutic monoclonal antibodies (mAbs) are one of the fastest growing classes of drugs, advances in biotechnology, antibody manufacturing, and scientific rationale have led to an increased focus over the last 20 years in bispecific proteins as an effective class of immunotherapy. Globally, there are five bispecific antibody (BsAb) drugs that have received market approval, including blinatumomab and emicizumab, and hundreds of candidates presently undergoing various stages of development and clinical trials. In addition to their application in the treatment of cancer, bispecific proteins have been evaluated as a potential treatment option for many other indications including chronic inflammatory diseases, autoimmunity, neurodegeneration, bleeding disorders, and infections[1].

Bispecific Protein Mechanisms of Action (MoA)

In therapeutics, there are four main mechanisms of action of bispecific proteins, including cell-bridging (ex - bridging target cell to T-cells), receptor/ligand blockers or activators (ex - receptor dimerization inhibitor or activator), cofactor mimetics, and homing (ex - blood brain barrier crosser). In this application note we focus on the application of bispecific proteins in targeting T-cell receptors (TCRs) to bridge T-cells to tumor antigens, but the core capabilities are applicable across all categories.

The typical MoA of a bispecific protein bridging a T-cell to a tumor cell involves one arm of the protein targeting a tumor specific antigen, such as peptide-HLA (also known as major histocompatibility complex (MHC) I molecules), and the other arm targeting a T-cell receptor, typically CD3e. Bridging of the target cell and the T-cell occurs through the bispecific protein, which leads to the formation of an immunological synapse, inducing T-cell activation and resulting in the release of cytotoxic proteins that lyse the target cell.

Immunocore ImmTAX® technology

Immunocore is a leading T-cell receptor biotechnology company that is developing a robust pipeline of transformative biologics built on their proprietary T-cell receptor (TCR) technology. This novel class of biologics,

called ImmTAX (Immune Mobilizing Monoclonal TCRs Against X disease), are bispecific molecules that combine an engineered TCR with a single chain antibody fragment (scFv) effector function (figure 1).

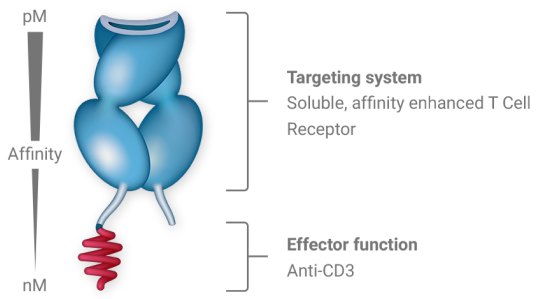


Figure 1: ImmTAC® molecules engage the natural T cell activation pathway via an anti-CD3 antibody fragment, scFv (single chain fragment variable), fused to the TCR via a flexible linker.

Typical antibody-based therapies are restricted to targeting cell surface or secreted proteins. By integrating high-affinity TCRs, ImmTAX molecules have the ability to recognize intracellular cancer or viral antigens presented by human leukocyte antigen (HLA) with ultra-high affinity and selectively, and redirect the immune system to kill these cancer and/or infected cells via an anti-CD3 immune-activating effector function (figure 2).

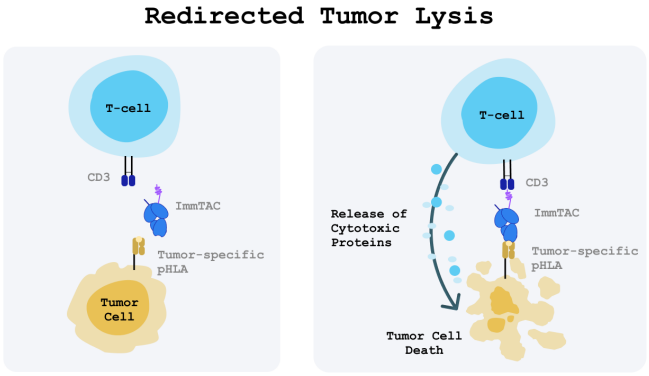


Figure 2: ImmTAC molecule targeting a T-cell receptor and tumor-specific pHLA, which induces the release of cytotoxic proteins upon bridging and results in tumor cell apoptosis.

Immunocore is currently using their ImmTAX technology to target a wide range of indications, given its potential to overcome the limitations of our natural immune system and current therapeutic approaches. In this application note, we analyze a subset of Immunocore's ImmTAX technology, called ImmTAC (Immune Mobilizing Monoclonal TCRs Against Cancer).

Characterizing Bispecific Proteins with SPR

To facilitate the development of these more complex biological therapies, highly selective and specific bioassays that adequately reflect the proposed MoA are needed from discovery, through development, and into production. Surface plasmon resonance (SPR) is a powerful tool that meets these needs and is well-suited for analyzing the complex interactions associated with bispecific proteins. Compared to limited techniques like ELISA, SPR is a direct binding technique which enables the detection and characterization of sequential binding events, which is necessary for the examination of dual-target specificities possessed by bispecific proteins in a single assay[2].

Advantages of SPR include:

- No need for additional labels, eliminating the introduction of another irrelevant binding event to account for and design around.
- Real-time binding data that enables deeper insight into the effect of multi-target binders on the kinetics rather than just the affinity.
- Precise control over the density of targets on the sensor surface to investigate the impact of avidity on the bispecific protein binding, which is an important factor to consider when moving to in-vivo.
- The ability to reveal the bioactivity of the bispecific protein and provide scientists with critical information about its MoA through the development pipeline.

Alto, a high-throughput SPR platform enabled with digital microfluidic (DMF) technology, has a number of advantages that make it ideal for SPR characterization of bispecific proteins. With the ability to discreetly control nano-liter sized droplets of each ligand and analyte, complex multi-step assays such as bridging and dual-binding assays are simple to implement and provide novel insights into therapeutic performance, while reducing consumption of precious samples by up to 200X. In addition, Alto's 16 independent channels provide the ability to simultaneously analyze multiple bispecific proteins in many different assay formats, while significantly reducing hands-on time with complete assay automation.

In this application note, we demonstrate the applicability of Alto in measuring a wide range of bispecific protein binding affinities and kinetics through characterization of

8 different ImmTAC molecules targeted to pHLA. We also confirm the accuracy of Alto compared to conventional SPR and highlight its numerous advantages in this important application.

Materials and Equipment

- Alto Instrument (ALTO16)
- Alto CBX Cartridge (KIN-CART-CBX-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Streptavidin Coupling Kit (ALTO-R-STREPTAVIDIN)
- Ligands and analytes: All bispecific proteins and pHLA ligands were supplied by Immunocore Ltd.

Method

Full experimental details are outlined on page 5.

Results & Discussion

The SPR binding assay for pHLA ligand and 8 bispecific ImmTAC molecules were performed in a single cartridge with Alto. The binding curves from each analyte are shown in Figures A-H in the Appendix . Single cycle kinetics (SCK) method was performed for this experiment given the inability to regenerate the interaction due to significant deactivation of the ligand, which was evident in both Alto and conventional SPR. The data was fit to a one-to-one binding kinetic model, except for analyte 1, where the KD was calculated using affinity analysis. All of the kinetic and affinity values are presented in Table 1, along with the results from a comparable assay performed on a conventional SPR system. The kinetic data reveals that the proteins exhibit a wide range of affinities and kinetics to the pHLA ligand. Alto was able to differentiate and obtain accurate kinetics and affinity values simultaneously for all 8 analytes in a single cartridge, across a dynamic range of 5 orders of magnitude in binding affinity. The experiment was completed in under 2 hours, requiring only 30 minutes of hands-on time for setup.

Accuracy of Alto Compared to Traditional SPR

The accuracy of the kinetic and affinity results obtained by Alto was supported by running the same set of samples in a traditional fluidics-based SPR instrument. The % difference between Alto kinetics values compared to traditional SPR are shown in Figures 3-5 and summarized in Table 2. These values are well within the expected variation when comparing between instruments, operators and methods. This accuracy in the absolute values of the kinetics and affinity also mean that the affinity and off-rate ranking is 100% in agreement with the ranking produced by the conventional SPR instrument.

Characterizing both affinity and kinetics of potential therapeutic candidates in-vitro is critical to ensuring that these molecules will demonstrate the desired selectivity

Table 1: Kinetic parameters measured for 8 analytes using Alto.

	Alto			Conventional SPR		
Analyte	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
1	-	-	775	-	-	792
2	5.61×10^{5}	2.15×10^{-2}	38.4	5.25×10^{5}	5.31×10^{-2}	101
3	9.40×10^{5}	2.24×10^{-3}	2.38	1.15×10^{6}	9.05×10^{-3}	7.89
4	1.04×10^{6}	2.20×10^{-3}	2.1	8.97×10^{5}	1.47×10^{-3}	1.64
5	9.8×10^{5}	2.10×10^{-4}	0.21	1.76×10^{6}	3.34×10^{-4}	0.19
6	2.09×10^{6}	1.14×10^{-4}	0.054	1.31×10^{6}	1.38×10^{-4}	0.105
7	1.35×10^{6}	5.62×10^{-5}	0.041	1.37×10^{6}	2.20×10^{-5}	0.0161
8	2.39×10^{6}	2.03×10^{-5}	0.0085	1.65×10^{6}	1.22×10^{-5}	0.0074

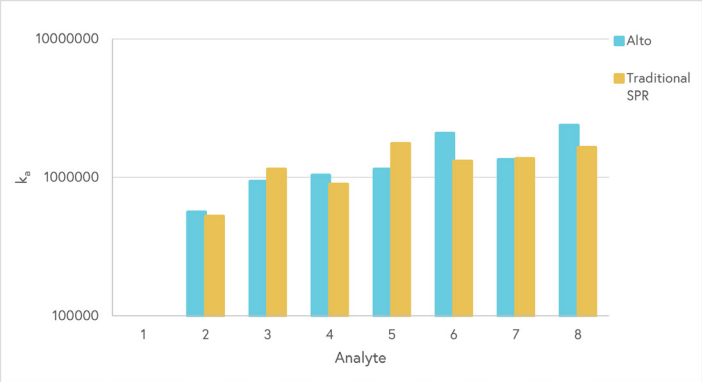


Figure 3: On-rate (ka) analysis between Alto and Traditional SPR. % difference of 26% across analytes.

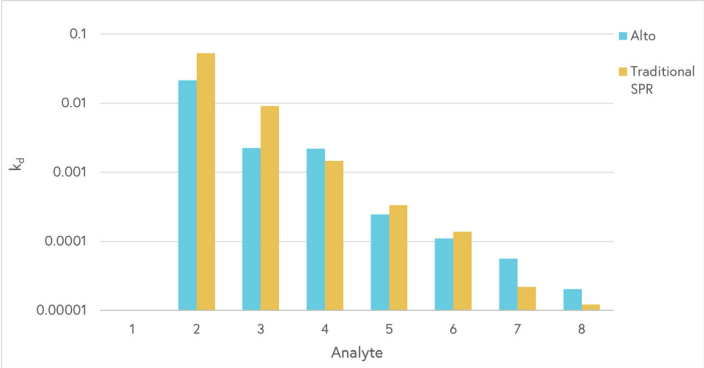


Figure 4: Off-rate (kd) analysis between Alto and Traditional SPR. % difference of 65% across analytes.

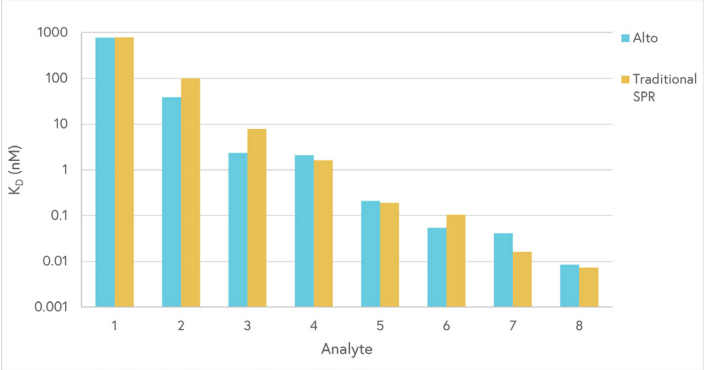


Figure 5: Affinity (KD) analysis between Alto and Traditional SPR. % difference of 49% across analytes.

Table 2: % difference between data generated by Alto compared to traditional SPR across all analytes.

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
% difference	26 %	65 %	49 %

and specificity in vivo. In this application, SPR assays conducted on Alto provided precise affinity and kinetic measurements for each of the 8 engineered bispecific proteins, providing validation of their varying TCR targeting abilities and providing insight into their overall MoA. This data is critical to the biologics discovery and development pipeline as the first stage of understanding their dual-target specificities and predicting their efficacy in redirecting T-cells in their function as a catalyst for tumor cell apoptosis. Alto's ability to provide high-quality binding data with a fraction of the time and sample volumes needed in traditional SPR systems demonstrates its unmatched capabilities in accelerating multi-analyte analysis and accelerating the bispecific protein discovery and development pipeline.

Conclusion

An analysis of 8 bispecific ImmTAC molecules with Alto demonstrated the platform's ability to characterize a wide range of affinities while providing comparable data to conventional SPR systems. All aspects of the experiment were automated by Alto using DMF technology, allowing all analysis to be conducted on a single cartridge with just a fraction of typical sample requirements, while requiring just 30 minutes of hand-on time with the instrument. The resulting data highlights Alto's ability to provide high-quality data while reducing time to answer, proving its ability to accelerate the development of new therapeutic classes such as bispecific ImmTAC molecules.

Experimental Details

Instrumentation

This label-free SPR assay was performed using Alto, the first and only digital microfluidic (DMF) powered SPR. Alto uses a cartridge-based, gold nanostructure sensor with 16 channels (8 reference channels and 8 active channels). The experimental method was designed remotely using Alto's cloud-based user portal, the Nicosystem, which was pushed to the instrument with a click of a button.

Method

First, a cartridge with carboxyl sensors (CBX) was loaded into Alto followed by dispensing of the cartridge fluid into the cartridge. Since Alto is an automated SPR instrument, all the reagents were pipetted into the cartridge wells as shown in Figure 6.

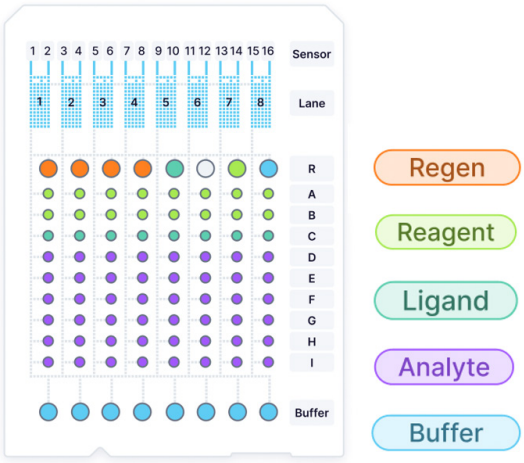


Figure 6: Layout of reagents pipetted into carboxyl cartridge.

After all the reagents were loaded into the cartridge, the experiment was initiated by selecting the "Run Method" command on Alto. All subsequent steps were automated by Alto.

Normalization of sensors with high and low RI droplets was first performed. The sensors were then cleaned with 10 mM HCl for 60s, followed by activation of 16 carboxyl sensors in the cartridge with 25 mM EDC/NHS for 5 mins. The capture molecule, streptavidin (100 ug/mL) in 10mM sodium acetate pH 5.0, was immobilized onto all 16 sensors for 5 mins, and all sensors were subsequently blocked with 1M Ethanolamine for 5 mins to quench the remaining active carboxyl groups. The biotinylated pHLA ligand at 5 µg/mL in PBS-T was passed over the 8 active channel for 5 minutes and captured by the streptavidin molecule.

The concentrations used for each analyte are different for each of the 8 bispecific proteins in order to facilitate optimal curve fitting over the wide range of affinities under investigation. For each analyte, 2uL of the stock concentrations were loaded into each analyte well, and the mixing of five 3-fold serial dilutions per analyte was automated by Alto. The concentrations of all stock solutions and dilutions are listed in Table 3. The 8 analytes were introduced in a single cycle kinetics (SCK) format with an association time of 240s, without dissociation or regeneration between each sample, starting from the lowest to highest concentration. A dissociation time of 2700s was set to run after the highest concentration. The sensor surface was regenerated with a 60s exposure of 10 mM Glycine-HCl pH 1.5, which resulted in a significant decrease in ligand activity. Consequently, only 1 cycle of data was used for analysis for each analyte.

A similar protocol was used on a traditional SPR instrument in order to compare the results to Alto, with some differences in the specific concentration ranges used.

Table 3: The stock concentrations (2uL) added into the sample wells and the 5 concentrations that were automatically generated by Alto to be used for the SPR assay

Analyte	[Sample] Pipetted	[Drop 1] (nM)	[Drop 2] (nM)	[Drop 3] (nM)	[Drop 4] (nM)	[Drop 5] (nM)
1	1.5 µM	6.17	18.5	55.5	167	500
2	0.5 µM	2.06	6.17	18.5	55.5	167
3	55 nM	0.23	0.68	2.04	6.11	18.33
4	55 nM	0.23	0.68	2.04	6.11	18.33
5	20 nM	0.082	0.25	0.74	2.22	6.67
6	20 nM	0.082	0.25	0.74	2.22	6.67
7	25 nM	0.102	0.31	0.93	2.77	8.33
8	25 nM	0.102	0.31	0.93	2.77	8.33

References

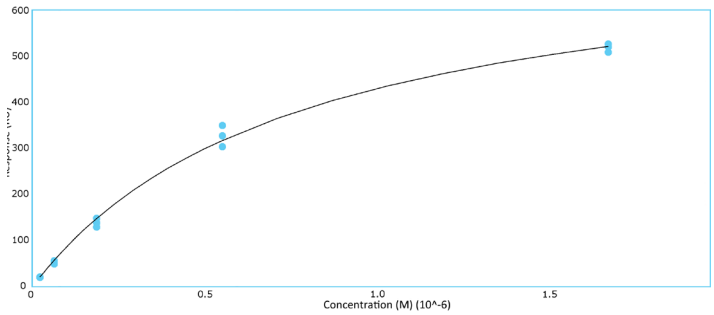
[1] Brinkmann, U., Kontermann, R.E. The making of bispecific antibodies. MAbs, 9:2, 182-212 (2017). doi: 10.1080/19420862.2016.1268307.

[2] Meschendoerfer, W., Gassner, C., Lipsmeier, F., Regula, J.T., Moelleken, J. SPR-based assays enable the full functional analysis of bispecific molecules. J Pharm Biomed Anal, 132, 141-147 (2017). doi: 10.1016/j.jpba.2016.09.028.

APPENDIX

Figures A-H: Binding of ImmTAC Analyte 1-8 to pHLA ligand.

A



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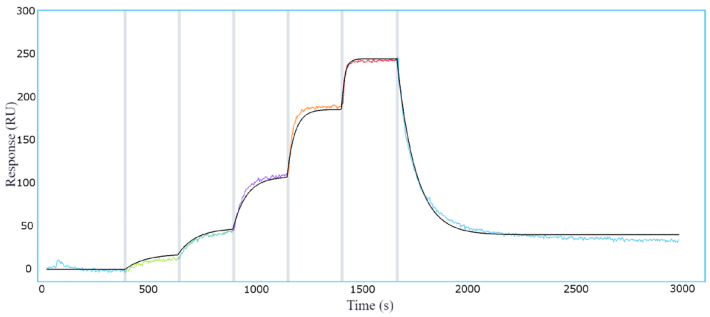

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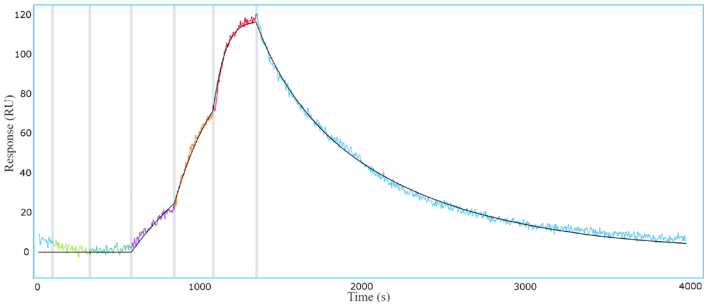

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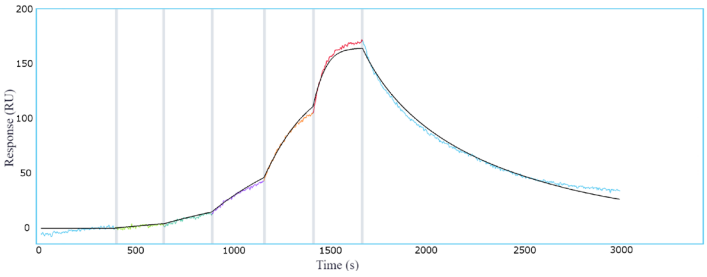

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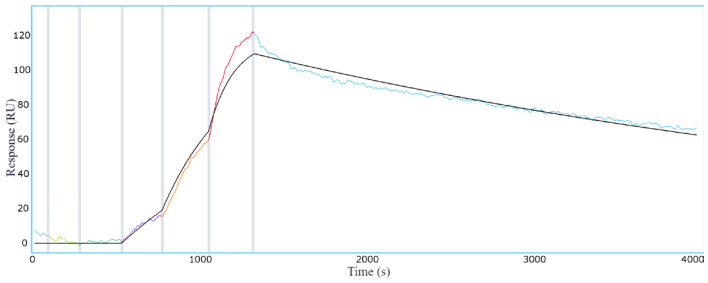

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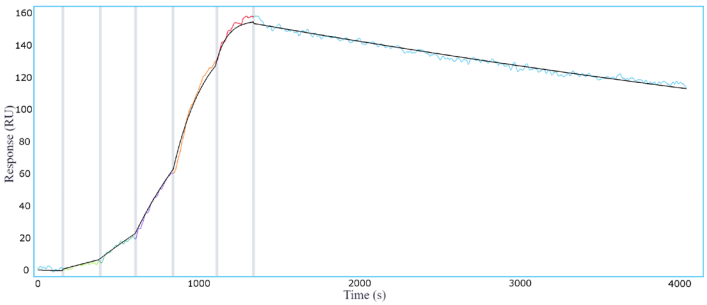

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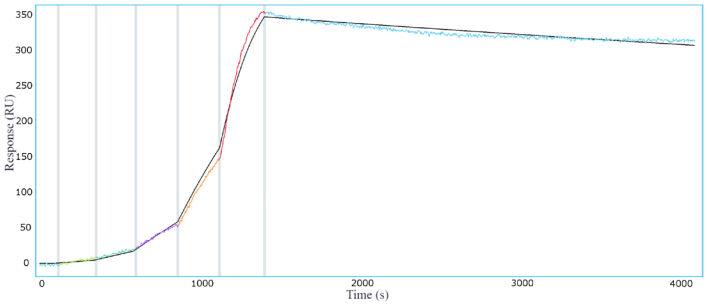

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