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1850 Millrace Drive, Suite 3A
Eugene, Oregon 97403
www.mitosciences.com

FlexPlex™ System Assay

Rev.0

FlexPlex Assays: Determining the molar amounts of OXPHOS complexes in human heart mitochondria and in human HepG2 cultured cells.

Introduction:
FlexPlex™ is a sandwich ELISA system for quantitatively and simultaneously measuring the levels of multiple metabolic enzymes in a tissue or cell extract. Each microplate can be configured in any desired combination from the available analytes.

This application note demonstrates the use of a FlexPlex assay configured to analyze each member of the mitochondrial inner membrane ATP generating OXPHOS system described in table I.

	OXPHOS component	Enzyme name	E.C.#
MSFX-1	Complex I	NADH dehydrogenase	1.6.5.3
MSFX-2	Complex II	Succinate dehydrogenase	1.3.5.1
MSFX-3	Complex III	Ubiquinone cytochrome c oxidoreductase	1.10.2.2
MSFX-4	Complex IV	Cytochrome c oxidase	1.9.3.1
MSFX-5	Complex V	ATP synthase	3.6.3.14
MSFX-8	NNT	Nicotinamide nucleotide transhydrogenase	1.6.1.2

Table I– Mitochondrial inner membrane enzyme systems OXPHOS and NNT.

Oxidative phosphorylation (OXPHOS) is an ATP generating metabolic pathway carried out by five enzyme complexes (I-V, Table I, Figure 1) in the mitochondrial inner membrane (for review see [1]). Electrons are transferred from NADH and succinate to the terminal electron acceptor oxygen by the first four OXPHOS complexes. As electrons flow through complexes I, III and IV protons are pumped from the mitochondrial matrix into the inner membrane space. In doing so, an electrochemical gradient is set up which, as the protons flow back through Complex V, provides the energy for the production of ATP synthesis by a rotational catalytic mechanism [2]. The enzyme nicotinamide nucleotide transhydrogenase (NNT), while not a part of oxidative phosphorylation, is also found in the mitochondrial inner membrane and uses the proton gradient formed by OXPHOS to generate NADPH on the matrix side of the membrane for biosynthetic reactions.

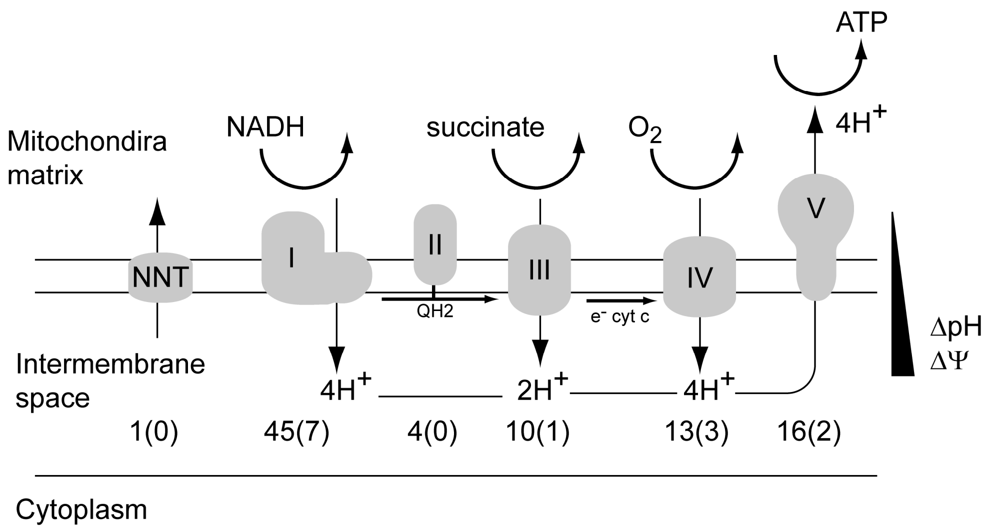

Figure I- Mitochondrial inner membrane OXPHOS and NNT. The electron transport system (I,II,III,IV) pumps protons into the intermembrane space in the mitochondrion as electrons are sequentially passed along its length to the terminal acceptor, oxygen. The proton build up is channeled back through Complex V synthesizing ATP and completing oxidative phosphorylation. The multisubunit enzyme OXPHOS system has dual genetic origin, shown are the number of subunits in each enzyme and in parentheses the number encoded by the mitochondrial DNA. NNT is an inner membrane protein which also uses the proton gradient to synthesize NADPH for biosynthetic purposes.

The organization of these enzymes in the inner membrane is intricate, Complexes I and II are likely monomeric while III, IV and V are dimers. Furthermore the possibility exists that these assemblies form supercomplexes which exist between III/IV and I/III/IV. Using a combination of spectrophotometric and electrophoretic approaches Schägger and Pfeiffer determined the OXPHOS stoichiometry as 1:1.3:3:6.7:3.5 for complexes I,II,III,IV,V in bovine heart mitochondria [3] with NNT present in a 1:1 stoichiometry with Complex I [4]. A number of earlier studies had determined the molar amounts of each enzyme in heart mitochondria [4-9] and are well summarized by Lenaz [10, 11]. Importantly these studies provide amounts in stoichiometries similar to the ratio proposed by Schägger and Pfeiffer. These amounts have been consolidated into a single molar relationship while adhering to this stoichiometric relationship in Table II.

	Stoichiometry	pmol / mg heart mitochondria	fmol / mg heart mitochondria
Complex I	1	0.12	120
Complex II	1.3	0.15	150
Complex III	3	0.35	350
Complex IV	6.7	0.78	780
Complex V	3.5	0.4	400
NNT	1	0.12	120

Table II– Determined ratio and approximate molar amounts of OXPHOS and NNT in heart mitochondria.

These multisubunit enzymes are structurally complex. Recombinant expression or purification in a completely functional state is not possible for each enzyme therefore we have established these approximate molar amounts for each complex in heart mitochondria in order to use a human heart mitochondrial sample as a standard to calibrate the FlexPlex assay system. In this way we shall determine the molar amounts and stoichiometric ratios in other human tissues and cultured human cell lines.

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Methods:

Mitochondrial preparation and cell culture. Human heart mitochondria were obtained from Analytical Biological Services, DE by tissue homogenization and differential centrifugation essentially as described by Smith [12] - the same methodology used to determine the stoichiometric and molar amounts described for bovine heart mitochondria [11]. HepG2 cells were cultured in HGDMEM medium containing 10% FCS, 100 µg/ml penicillin and 100 U/ml streptomycin on Purecoat amine coated flasks (BD) to confluence.

Sample detergent extraction. Human heart mitochondria or cell culture pellets were lysed according to the FlexPlex standard protocol by adding 9 volumes of extraction buffer to 1 volume of sample pellet. Protein concentration was determined for extracted samples by BCA method (Pierce). Human heart mitochondria detergent extract was used as a standard sample. A two-fold dilution series of this standard was prepared for OXPHOS complexes I-V and NNT in incubation buffer according to the provided dilution series guidelines. A dilution series of standard sample 0.38-10 µg/50 µL well (i.e. 0.75-200 µg/mL) was prepared. Cell culture pellet detergent extract was the experimental sample of unknown OXPHOS composition. Standards and unknowns were loaded into the FlexPlex microplate as shown in Table III and Figure 2.

Assay and Analysis. The assay was performed according to the provided FlexPlex protocol ensuring to add only the appropriate 1X detector antibody to the corresponding microplate well (step C4) and only the appropriate HRP label to the corresponding microplate well (step C6). Data was collected using a SPECTRAmax plus 384 microplate reader (Molecular Devices) using the bundled software SoftMax Pro 4.8 in \Assays\Kinetic ELISA assays\. Readings were made at 600 nm for 15 minutes with a 20 second interval and a three second shake between readings. Using the software, a four-parameter fit was applied to the standard sample series and unknowns were interpolated as pmol/mL from this standard curve.

Results:

Plate setup- A two-fold dilution series of human heart mitochondria detergent extract 0-038-10 µg / 50 µL well (i.e. 0.75-200 µg/mL) was created by dilution in the incubation buffer as described in the FlexPlex protocol and shown below in Figure 2.

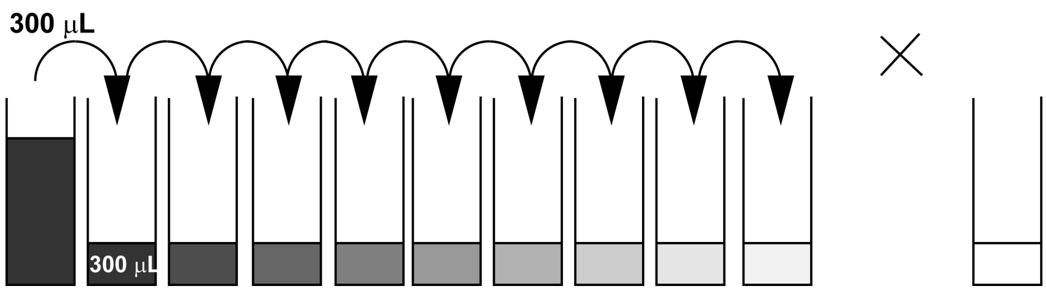


Figure 2- A two-fold dilution of the standard sample was made in incubation buffer. Nine dilutions were necessary to cover the entire ranges of all assays used. A tenth tube contained no sample and was used as a null or buffer control.

Microplate strips were labeled FX1 (Complex I strips 1-2), FX2 (Complex II strips 3-4), FX3 (Complex III strips 5-6), FX4 (Complex IV strips 7-8), FX5 (Complex V strips 9-10) and FX8 (NNT strips 11-12). The appropriate standard samples were added to the odd numbered microplate strips and experimental samples were added to even numbered strips according to Table III and Figure 3.

Wells G through A		fmol in wells G-A
0.15-10 µg HHM	Complex I	0.018-1.2
0.15-10 µg HHM	Complex II	0.0225-1.5
0.038-2.5 µg HHM	Complex III	0.0133-0.875
0.15-10 µg HHM	Complex IV	0.117-7.8
0.15-10 µg HHM	Complex V	0.06-4
0.038-2.5 µg HHM	NNT	0.00456-0.3

Table III– A human heart mitochondria standard was loaded into the FlexPlex microplate in the range described above which is specified by each assay's accompanying technical datasheet. The molar amounts of each enzyme in that sample were calculated and used to construct the standard curve.

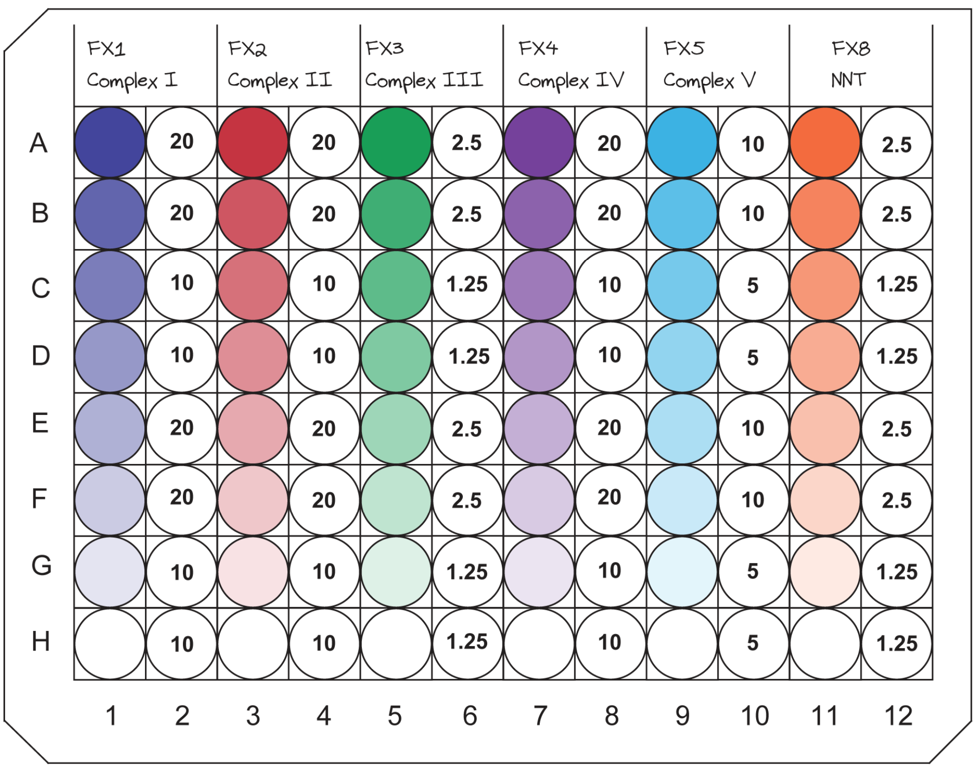
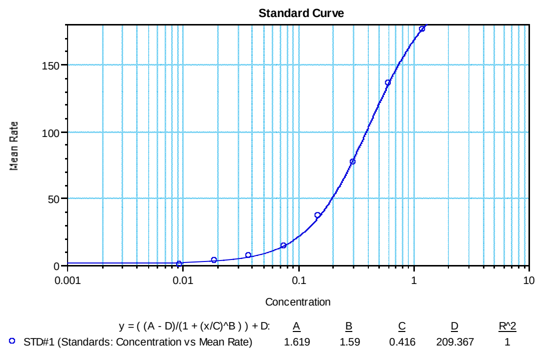


Figure III – Assays are supplied with a blank plate map. This plate map has been completed for this experiment to identify the standard dilutions loaded for each assay (colored and described in Table III). Numbers identify experimental samples - a HepG2 cell lysate, which was added at the microgram/well amounts indicated to be interpolated from the standard curve.

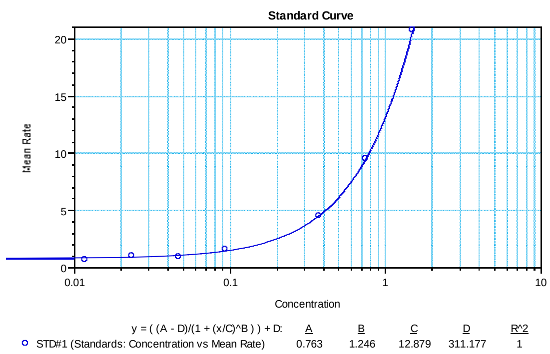
Standards curves and interpolated unknowns- Standard curves were drawn by, and the data from unknowns was determined directly by the microplate instrument software SoftMax Pro 4.8 without further manipulation. Other microplate software packages perform similar calculations; alternatively raw data can be exported to GraphPad Prism, or other graphing software, where a 4-paramteter fit should be made to standard data points and unknowns interpolated from this standard curve fit.

FX1 Complex I



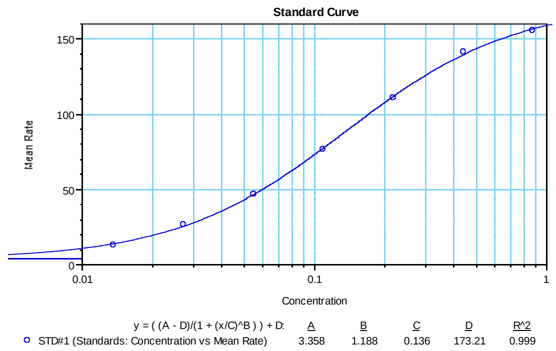
Sample	Wells	Rate	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result [fmol/mg]
Un01	A2	40.347	0.165	0.170	0.008	4.8	50.0	8.518
	B2	43.857	0.176					
Un02	C2	22.265	0.104	0.105	0.001	1.4	100.0	10.498
	D2	22.852	0.106					

FX2 Complex II



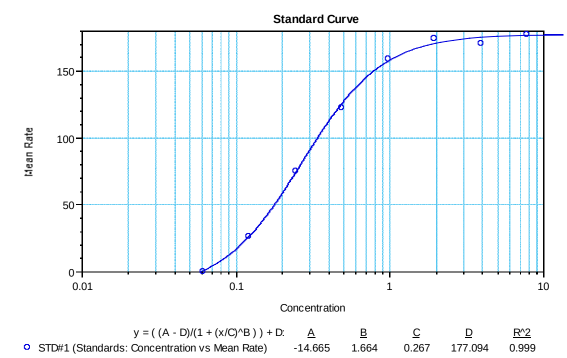
Sample	Wells	Rate	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result [fmol/mg]
Un01	A4	6.112	0.501	0.460	0.058	12.6	50.0	23.004
	B4	5.059	0.419					
Un02	C4	3.450	0.286	0.275	0.016	5.7	100.0	27.527
	D4	3.196	0.264					

FX3 Complex III



Sample	Wells	Rate	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result [fmol/mg]
Un01	A6	81.055	0.118	0.121	0.004	3.7	400.0	48.466
	B6	83.656	0.124					
Un02	C6	53.594	0.066	0.066	0.000	0.3	800.0	52.610
	D6	53.752	0.066					

FX4 Complex IV



Sample	Wells	Rate	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result [fmol/mg]
Un01	A8	156.829	0.965	0.922	0.060	6.5	50.0	46.110
	B8	153.862	0.880					
Un02	C8	133.658	0.559	0.546	0.018	3.3	100.0	54.646
	D8	130.985	0.534					

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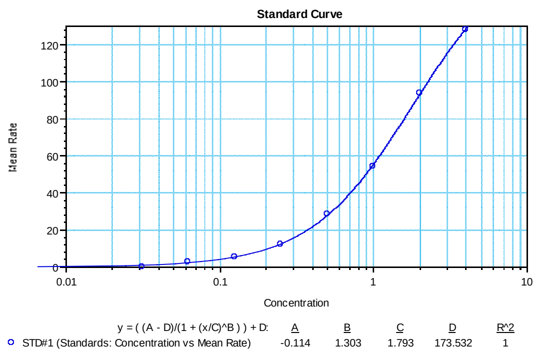
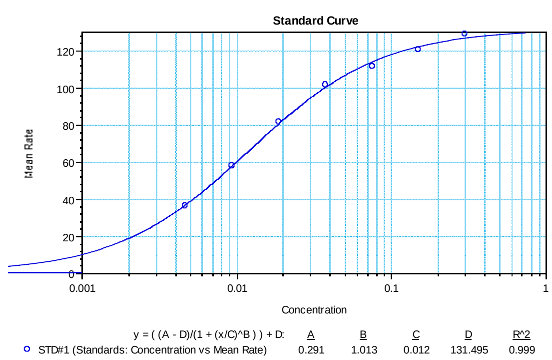
FX5 Complex V



Sample	Wells	Rate	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result [fmol/mg]
Un01	A10	141.957	5.687	4.634	1.489	32.1	100.0	463.396
	B10	123.387	3.581					
Un02	C10	90.349	1.912	1.873	0.055	3.0	200.0	374.566
	D10	87.982	1.834					

FX8 NNT



Sample	Wells	Rate	Result	MeanResult	Std.Dev.	CV%	Dilution	Adj.Result [fmol/mg]
Un01	A12	91.369	0.027	0.026	0.001	4.1	400.0	10.440
	B12	89.710	0.025					
Un02	C12	66.730	0.012	0.012	0.001	7.6	800.0	9.307
	D12	63.138	0.011					

Conclusions:

When a human heart mitochondrial sample was used as the assay standard sample the molar amounts of enzymes within this sample could be used to calibrate the signals. In this way the molar amounts of each enzyme can be determined in an unknown sample. In this example a human hepatoma cell hepG2 was used. Enzyme amounts in HepG2 were interpolated from within the standard curves with low coefficient of variation. In general OXPHOS complexes were found to be approximately ten fold lower in HepG2 cell line than in human heart per mg, consistent with muscle's greater need for ATP and hence oxidative phosphorylation. To most easily compare the OXPHOS stoichiometry in HepG2 with other samples, Complex I was set to 1 as shown in Table IV. Comparing this ratio Complex I and NNT were identified to be in equimolar amounts in the HepG2 sample. This is consistent with our hypothesis that these two enzymes are equimolar in heart tissue. Assembled Complexes II and III enzymes were found to be in approximately two fold higher relative amounts in the liver derived HepG2 cell line, which is consistent with our Western blotting analysis and also a FlexPlex analysis of mitochondria derived from liver and heart. Most strikingly HepG2 cells contained a much larger relative amount of Complex V, ATP synthase. The actual molar amount determined in HepG2 was approximately equivalent to the heart sample per mg. Once again this is consistent with our previous results comparing HepG2 cells and heart tissue by Western, FlexPlex and Complex V activity.

	fmol / mg heart mitochondria	Stoichiometry Hepg2	For comparison: stoichiometry heart
Complex I	9.5±1	1	1
Complex II	25.2±3	2.7	1.3
Complex III	50.5±3	5.3	3
Complex IV	50.4±6	5.3	6.7
Complex V	419±63	44	3.5
NNT	9.9±0.8	1	1

Table IV- the molar amounts of the OXPHOS complexes and NNT as determined by this experiment in HepG2 cells. The relative stoichiometry of the OXPHOS complexes was calculated from these amounts and compared to the stoichiometric arrangement in heart mitochondria.

It is unclear why HepG2 cells would contain enhanced levels of Complex V. It is possible that elevated levels of this enzyme operating as an ATP driven proton pump are necessary to maintain the high mitochondrial membrane potential observed in glycolytic transformed liver cells. Alternatively, while nuclear OXPHOS and mitochondrial OXPHOS gene products have been reported to be coordinately regulated, the possibility exists that in HepG2 cells this enzyme is predominantly in the soluble F1 form while the F0 domain containing the two mtDNA encoded components are regulated separately along with the other mtDNA encoded elements of Complexes I, III and IV.

In summary using a rationale of this kind, a sample of known native enzyme composition can be used to calibrate the standard curves and determine molar amounts of enzyme in an unknown sample. In the future these molar ranges, a human heart mitochondria sample, or the Hepg2 sample as defined here, could be used as standards to determine the molar amounts of these enzymes in other tissue types, cell lines or patient cells.

1. Schultz, B. E. & S. I. Chan, Structures and proton-pumping strategies of mitochondrial respiratory enzymes, Annu Rev Biophys Biomol Struct 30 (2001) 23-65.
2. Noji, H. & M. Yoshida, The rotary machine in the cell, ATP synthase, J Biol Chem 276 (2001) 1665-1668.
3. Schagger, H. & K. Pfeiffer, The ratio of oxidative phosphorylation complexes I-V in bovine heart mitochondria and the composition of respiratory chain supercomplexes, J Biol Chem 276 (2001) 37861-37867.
4. Capaldi, R. A., Arrangement of proteins in the mitochondrial inner membrane, Biochim Biophys Acta 694 (1982) 291-306.
5. Blair, P. V., T. Oda & D. E. Green, Studies on the Electron Transfer System. Liv. Isolation of the Unit of Electron Transfer, Biochemistry 2 (1963) 756-764.
6. Green, D. E. & D. C. Wharton, Stoichiometry of the Fixed Oxidation-Reduction Components of the Electron Transfer Chain of Beef Heart Mitochondria, Biochem Z 338 (1963) 335-348.
7. Hackenbrock, C. R., B. Chazotte & S. S. Gupte, The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport, J Bioenerg Biomembr 18 (1986) 331-368.
8. King, T. E., K. S. Nickel & D. R. Jensen, Iron, Copper, Cytochrome, and Lipid Contents of Heart Muscle Preparation and Heart Mitochondria, J Biol Chem 239 (1964) 1989-1994.
9. Murray, J., R. Gilkerson & R. A. Capaldi, Quantitative proteomics: the copy number of pyruvate dehydrogenase is more than 10(2)-fold lower than that of complex III in human mitochondria, FEBS Lett 529 (2002) 173-178.
10. Lenaz, G. & M. L. Genova, Kinetics of integrated electron transfer in the mitochondrial respiratory chain: random collisions vs. solid state electron channeling, Am J Physiol Cell Physiol 292 (2007) C1221-1239.
11. Pallotti, F. & G. Lenaz, Isolation and subfractionation of mitochondria from animal cells and tissue culture lines, Methods Cell Biol 65 (2001) 1-35.
12. Smith, A. L., Preparation and properties and conditions for assay of mitochondria: Slaughterhouse material small scale., Methods Enzymol 10 (1967) 81-86.

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