

Data Analysis for G Protein-Coupled Receptor Assays Using Corning® Epic® Technology



SnAPPShots

A brief technical report
from the Corning
Applications Group

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Introduction

G protein-coupled receptors (GPCRs) constitute one of the largest therapeutic target classes being investigated for novel drug discovery. Furthermore, approximately 40 to 50% of all pharmaceutical drugs act on these receptors. Label-free assays offer a new approach to identifying compounds that target GPCRs. Activation of GPCRs results in the translocation of multiple signaling molecules throughout the cell, and in many cases leads to widespread cytoskeletal reorganization. This dynamic mass redistribution (DMR) can be monitored in living cells using Corning Epic technology; a label-free and noninvasive system that uses resonant waveguide grating biosensors. Advantages of label-free measurements using Epic technology include (i) a single assay platform for all G-protein signaling pathways¹⁻⁴; (ii) a real-time optical response to monitor the full cellular response following GPCR activation; and (iii) sensitivity to detect responses from endogenous receptors expressed in native cells.

Microplates incorporating biosensors in every well can be used for assays on either the Epic Reader or the PerkinElmer EnSpire® Multimode Plate Reader with Epic technology. GPCR assays run on either reader are highly comparable in terms of pharmacological measurements and overall assay robustness.⁵ In this document, the process for analyzing the optical response data generated using Epic technology is described, in order to obtain pharmacology (EC_{50}/IC_{50}) and robustness measurements (Z'). Assays were performed using agonists and antagonists targeting the endogenous β_2 -adrenergic receptor (β_2 -AR) in A431 cells and endogenous muscarinic (M1) and adenosine (A1) receptors in HEK 293 cells. These targets were chosen to cover the three important coupling mechanisms utilized by GPCRs; G_s (β_2 -AR), G_i (A1) and G_q (M1). Data analysis methods described here will be applicable to all classes of cell-based assays run using Epic technology.

Materials and Methods

Cells

A431 cells (Cat. No. CRL-1555) and HEK 293 cells (Cat. No. CRL-1573) were obtained from American Type Culture Collection (Manassas, VA). Both cell lines were maintained in Dulbecco's Modified Eagle Medium (Cat. No. 10313) supplemented with 10% heat-inactivated Fetal Bovine Serum (Cat. No. 10082) and 2 mM L-Glutamine (Cat. No. 25030). Assay buffer was Hank's Balanced Salt Solution (Cat. No. 14025) containing 20 mM HEPES (Cat. No. 15630) and 1% Dimethyl Sulfoxide (DMSO) (Cat. No. D4540). All cell culture reagents were purchased from Invitrogen (Carlsbad, Calif.) except for DMSO, which was purchased from Sigma-Aldrich (St. Louis, MO).

Reagents

Isoproterenol hydrochloride, Propranolol hydrochloride, N⁶-Cyclopentyladenosine (N⁶-CPA), Carbamoylcholine chloride (Carbachol) and Atropine were all purchased from Sigma-Aldrich. DPCPX was obtained from Tocris Bioscience (Ellisville, MO).

Epic Assay Procedures

Cell Seeding

Stock subcultures of A431 and HEK 293 cells were grown to ~80% confluency on standard tissue culture treated (TCT) growth surfaces for 3 days then harvested using trypsin. Harvested cells were diluted in complete growth medium and counted. A431 cells were then seeded into Epic 384 well uncoated cell assay microplates (Cat. No. 5040) at a density of 20K cells/well while HEK 293 cells were seeded into Epic 384 well fibronectin coated cell assay microplates (Cat. No. 5042) at 18K cells/well. All microplates were seeded using a Thermo Scientific® Multidrop Combi cell culture dispensing system. The seeded microplates were allowed to sit at room temperature for 30 minutes to allow the cells to uniformly settle on the growth surface of the well and then incubated prior to beginning the assay (average 18 to 22 hours in a 37°C/5% CO₂ incubator).

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Buffer Exchange

After overnight culture, the growth medium was replaced with assay buffer containing 1% DMSO using the Molecular Devices Aquamax® DW4 automated plate washer. The plate was then stored at room temperature for 1 to 2 hours prior to running the assay to allow for recovery from the wash procedure.

Epic® Label-Free Measurement

The assay microplate was loaded into the Epic reader and a baseline measurement was taken for 5 minutes. For agonist assays, compounds were added to the cells in assay buffer and then a kinetic read was taken for either 30 minutes (HEK 293 cells) or 60 minutes (A431 cells). For antagonist assays, antagonist compounds were first added to the cells in assay buffer followed by an initial kinetic read of 30 to 60 minutes. Subsequently, agonists were added to the cells in assay buffer and a second kinetic read of either 30 minutes (HEK 293 cells) or 60 minutes (A431 cells) was performed. All compound additions were performed using a CyBio CyBi®-Well pipetting system. Each of the assays was performed at least three times.

Data Analysis

DMR response values were obtained using Epic Data Analysis software. Dose response curves and optical response profiles were generated using Graphpad Prism® software.

Results and Discussion

Optical response profiles of GPCR activation generated using Epic technology are highly characteristic of the coupling mechanism activated by the receptor. Figure 1 shows the DMR agonist profiles for each of the three principal coupling mechanisms utilized by GPCRs; G_s , G_q and G_i . The G_s -coupled profile of Isoproterenol is distinctive, exhibiting an early negative DMR (N-DMR) phase that quickly transitions into a prolonged positive DMR (P-DMR) phase. In contrast, the G_q - and G_i -coupled responses of Carbachol and N^6 -CPA are very similar beginning with a rapid P-DMR peak followed by a slightly slower N-DMR phase which typically plateaus after 15 to 20 minutes. It is usually neces-

sary to utilize pathway-specific inhibitors, such as Pertussis Toxin (G_i -inhibitor), to distinguish between G_q - and G_i -coupled responses. Confirmation of the G_s -coupled response can also be obtained using Cholera Toxin. At present, the most effective G_q -inhibitor (YM-254890) is not commercially available; therefore, G_q -coupled responses can only be inferred if Pertussis or Cholera Toxin fail to inhibit the response. Evaluation of toxin effects, together with analysis of G12/13-coupled responses was studied by Schröder et al. (4).

Dose response curves (DRCs) can be readily obtained from the optical response profiles to measure compound pharmacology. Figure 2 illustrates different approaches to analyzing a dose series of Isoproterenol in A431 cells. Using the difference in response values between the minimum N-DMR response (Min) and the end-point of the P-DMR response (E), a DRC of maximum signal response is generated, with an EC_{50} of 62.8 μ M. Alternatively, if the baseline measurement (B) is subtracted from E, a DRC with an EC_{50} of 55.8 μ M is observed but the overall assay window at the highest doses is reduced by 20 to 30 pm. Based upon the highly similar EC_{50} values, either approach is valid when analyzing data for G_s -coupled responses. Similar approaches to data analysis can be used for the G_q -coupled responses, as shown for Carbachol in HEK 293 cells in Fig. 3. Carbachol DRCs can be plotted from B to the initial P-DMR peak (Max), resulting in an EC_{50} of 5.23 μ M. Another possibility is to plot the DRC using B and the endpoint at E, which results in a similar EC_{50} of 8.60 μ M but the assay window is attenuated by almost 50%. Our recommendation when developing assays for G_q -coupled responses is to use the data values at B and Max in order to maximize the assay window. Based upon the almost identical optical response profiles of G_q - and G_i -coupled responses, the same approach should be used to generate DRCs for G_i -coupled agonists. Plotting the data for N^6 -CPA, the EC_{50} value using B and E is right-shifted approximately 5-fold in comparison to the EC_{50} value derived using B and Max (Table 1). This further supports the rationale for plotting DRCs using B and Max with G_q - and G_i -coupled responses; larger assay window and more potent pharmacology.

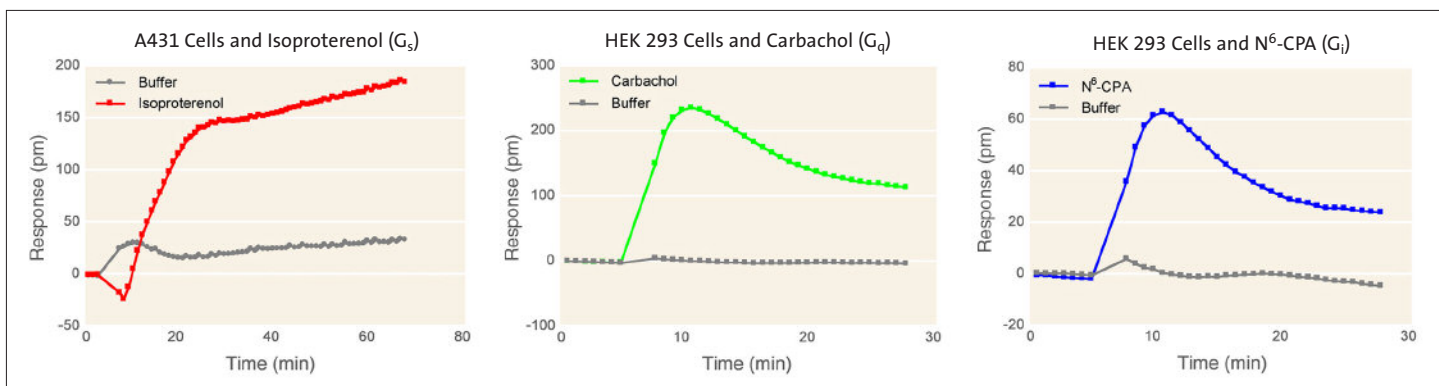


Figure 1. Optical response profiles are characteristic of the signaling pathways activated by GPCRs. Endogenous response profiles were obtained for Isoproterenol (G_s -coupled agonist), Carbachol (G_q -coupled agonist) and N^6 -CPA (G_i -coupled agonist) in either A431 or HEK 293 cells. All response profiles include the buffer control (grey data points).

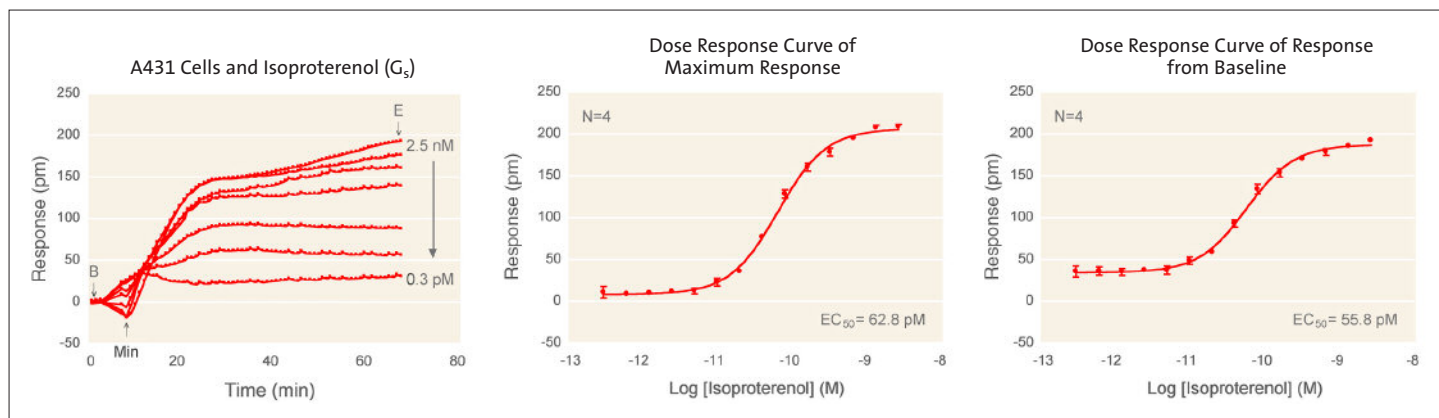


Figure 2. Agonist dose response curves are readily obtained from optical response profiles. A dose range of Isoproterenol (G_s -coupled agonist) in A431 cells was analyzed to generate dose-response curves. In the center panel, the curve is plotted using the response value at the minimum N-DMR point (Min) subtracted from the end point (E) for each dose of agonist. Alternatively, the curve can be plotted by subtracting the baseline (B) value from E (right panel).

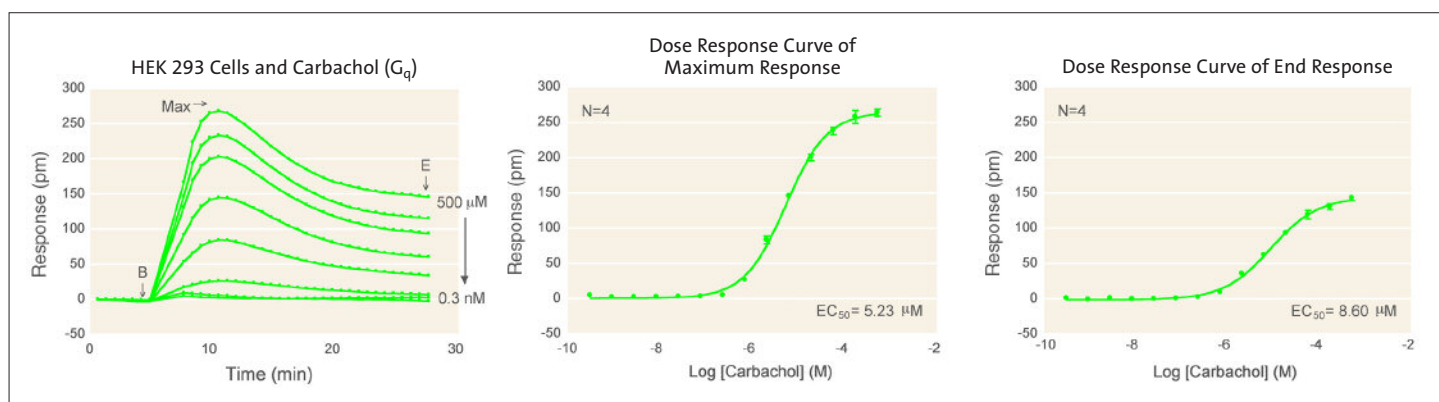


Figure 3. Dose response curves for G_q - and G_i -coupled agonists are also readily obtained from optical response profiles. A dose range of Carbachol (G_q -coupled agonist) in HEK 293 cells was analyzed to generate dose-response curves. In the center panel, the curve is plotted using the response value at the baseline (B) subtracted from the peak response value (Max). The DRC can also be plotted by subtracting B from the end point (E) (right panel).

Once reproducible pharmacology has been demonstrated, it is necessary to establish that assay conditions are highly robust. We determined the robustness (Z') of each agonist by measuring the peak response at an EC_{90} dose against the response to cells receiving assay buffer alone (Fig. 4 and Table 1). For each agonist, the response was plotted using the same approach as described for DRCs; G_s -coupled (use Min and E), G_q - and G_i -coupled (use B and Max). Buffer responses were measured at the same time points. When

GPCR assays have been fully optimized, it is typical to obtain Z' values of 0.7 or above, demonstrating the feasibility of moving into High-throughput screening.

Epic® technology can also be used to measure antagonist responses and these can be generated in the same well as agonist data using a dual addition strategy.⁶ Compounds are first added to the microplate to measure agonist responses. Immediately following completion of the agonist read, $EC_{80/90}$ dose of an agonist targeting the receptor of interest

Table 1. Summary of agonist pharmacology (EC_{50}) using Epic technology. EC_{90} values were used for robustness data in Figs. 4 and 5.

Cells	A431	HEK 293	HEK 293
Agonist	Isoproterenol	Carbachol	N^6 -CPA
Coupling	G_s	G_q	G_i
EC_{50}	Min and E: 63 pM B and E: 56 pM	B and Max: 5.2 μ M B and E: 8.6 μ M	B and Max: 49 nM B and E: 234 nM
EC_{90}	1 nM	30 μ M	1 μ M

is added to all wells, and a second scan is performed. Any compounds which act as antagonists will be revealed by inhibition of the agonist response in the second read. Benefits of the dual addition strategy include (i) discrimination between neutral antagonists (no activity in agonist read) and antagonists exhibiting partial agonist activity (some activity in agonist read), and (ii) reduced costs for assays by combining responses in a single well. Figures 5 and 6 illustrate the approach taken to analyzing pharmacology (IC_{50}) data for neutral antagonists targeting G_s - (Propranolol) and G_q -coupled (Atropine) receptors, respectively. In each case, the

antagonist is first added and read for either 60 minutes (G_s) or 30 minutes (G_q), resulting in a flat response. Agonists were then added at EC_{90} doses (Table 1) and read for the same period of time. Dose-dependent inhibition of the agonist response is observed for both compounds, and DRCs are plotted using the same approach as described for agonists. As with agonist assays, G_q - and G_i -coupled responses are very similar and should be analyzed in the same way. The IC_{50} value for DPCPX, targeting the G_i -coupled A1 receptor is shown in Table 2.

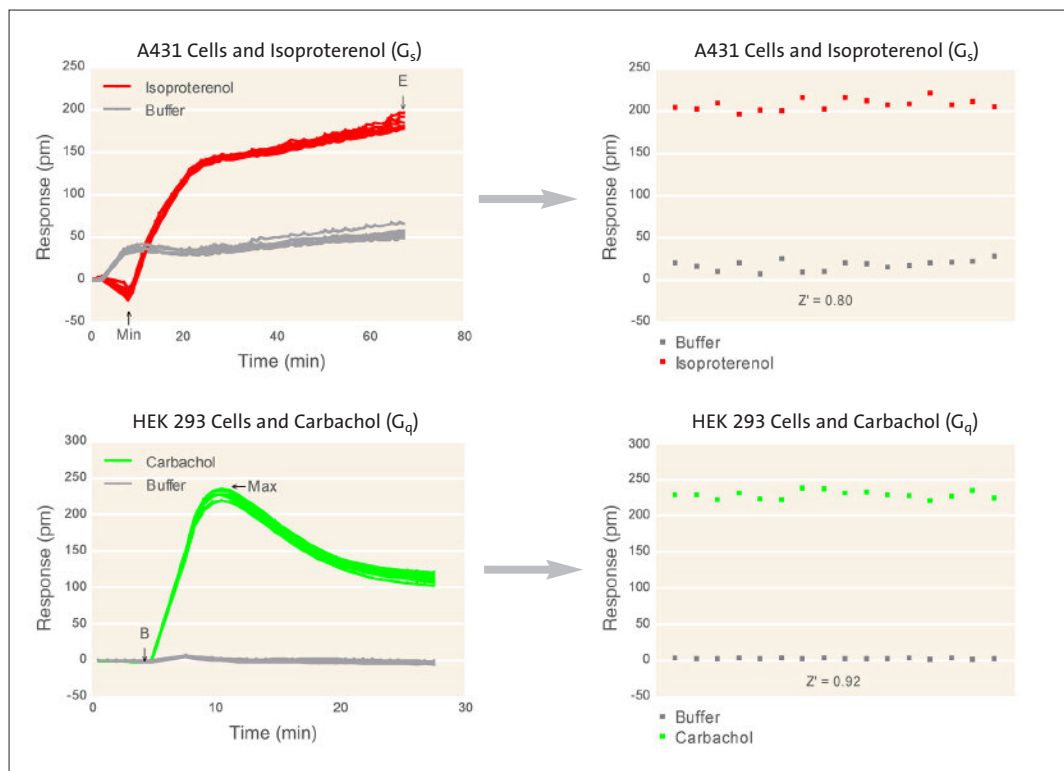


Figure 4. Assay robustness can be calculated from optical response profiles. Isoproterenol and Carbachol were tested at EC_{90} doses and the DMR profiles are shown in the left panels. Robustness plots (right panels) show the response from each individual well treated with either assay buffer alone (grey squares) or buffer plus agonist (colored squares).

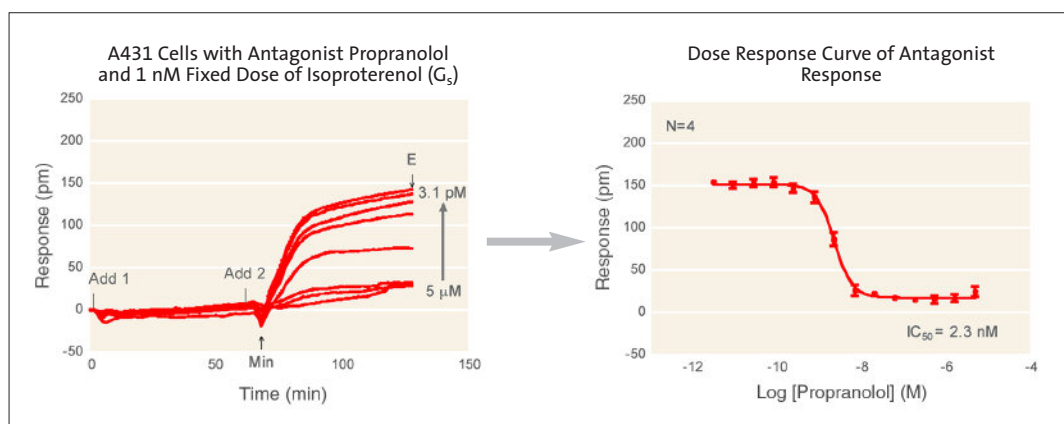


Figure 5. Optical profiles of antagonist responses can be analyzed to generate an inhibition dose response curve. A431 cells were first treated with a dose series of the G_s -coupled antagonist Propranolol (Add 1) for 60 minutes and then stimulated with Isoproterenol at an EC_{90} dose (Add 2) for a further 60 minutes (left panel). The inhibition dose response curve (right panel) was plotted using the response value at the minimum N-DMR point (Min) subtracted from the end point (E).

In conclusion, the approaches to analyze Epic® technology data described here, will enable users to develop GPCR agonist and antagonist assays. Furthermore, the principles of data analysis are applicable to the DMR response profiles obtained in other classes of assays including ion channels, receptor-tyrosine kinases and transporters.

Conclusions

- ▶ Epic technology data analysis tools can be used to generate dose response curves for GPCR agonist and antagonist pharmacology.
- ▶ Kinetic optical profiles can be analyzed at different time points to determine how pharmacology changes over time course of response.
- ▶ GPCR agonist robustness (Z') can be readily generated using Epic software.
- ▶ Epic software generates optical profiles that are characteristic of the GPCR coupling mechanism.

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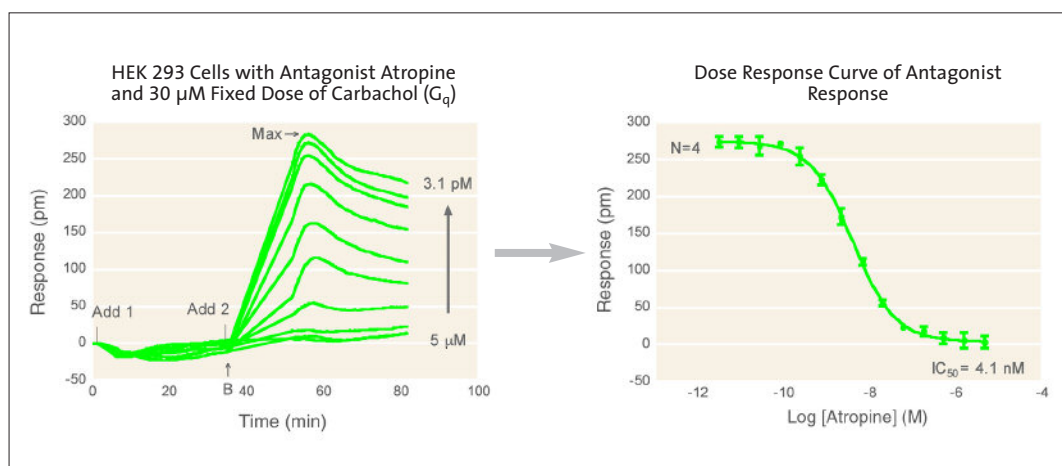


Figure 6. Inhibition dose response curves for G_q - and G_i -coupled antagonists are also readily obtained from optical response profiles. HEK 293 cells were first treated with a dose series of the G_q -coupled antagonist Atropine (Add 1) for 30 minutes and then stimulated with Carbachol at an EC_{90} dose (Add 2) for a further 30 minutes (left panel). The inhibition dose response curve (right panel) was plotted using the response value at the baseline (B) subtracted from the peak response value (Max).

Table 2. Summary of antagonist pharmacology (IC_{50}) using Epic Technology.

Cells	A431	HEK 293	HEK 293
Antagonist	Propranolol	Atropine	DPCPX
Agonist	Isoproterenol	Carbachol	N^6 -CPA
IC_{50}	2 nM	4 nM	250 nM Cells

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