HTRF IP-One assay: A robust and stable platform for high throughput screening of Gαq-coupled GPCRS.

This technical note presents data showing the stability and ease-of-use of the assay, optimal choice for robotic high throughput screening campaigns.

Abstract

Revvity HTRF® IP-One assay is a highly reproducible, reliable technique for downstream measurement of Gαq-coupled GPCR receptor activation. This technical note presents data showing the stability and ease-of-use of the assay, making it a flexible and optimal choice for robotic high throughput screening campaigns.

Introduction

High-throughput screening (HTS) involves the screening of chemical libraries of millions of compounds, generally at a rate of 100,000 compounds a day. Assay performance and robustness is a primary consideration before implementing an assay on an HTS platform. However, ease of use, reagent stability and a quality assay signal are essential as well for a high quality screen, especially at today's throughput levels.

Cell-based functional assays used for compound screening and lead optimization play an important role in drug discovery for G-protein coupled receptors (GPCRs). Commonly used calcium assays assess the activity of GPCRs by measuring the calcium released, which is inherently transient, resulting in a very short temporal window to actually measure it. Commercially available assays can be adapted to high-throughput environments, but have several limitations and require expensive dedicated instrumentation to record the rapid changes in intracellular free calcium concentration over time.

In contrast, Revvity IP-One kit is designed to be robust, reliable, and easy to use. As shown in this technical note, the assay's reagent and signal stability eliminate throughput limitations, and provide flexibility and consistency in use well beyond calcium flux technologies.

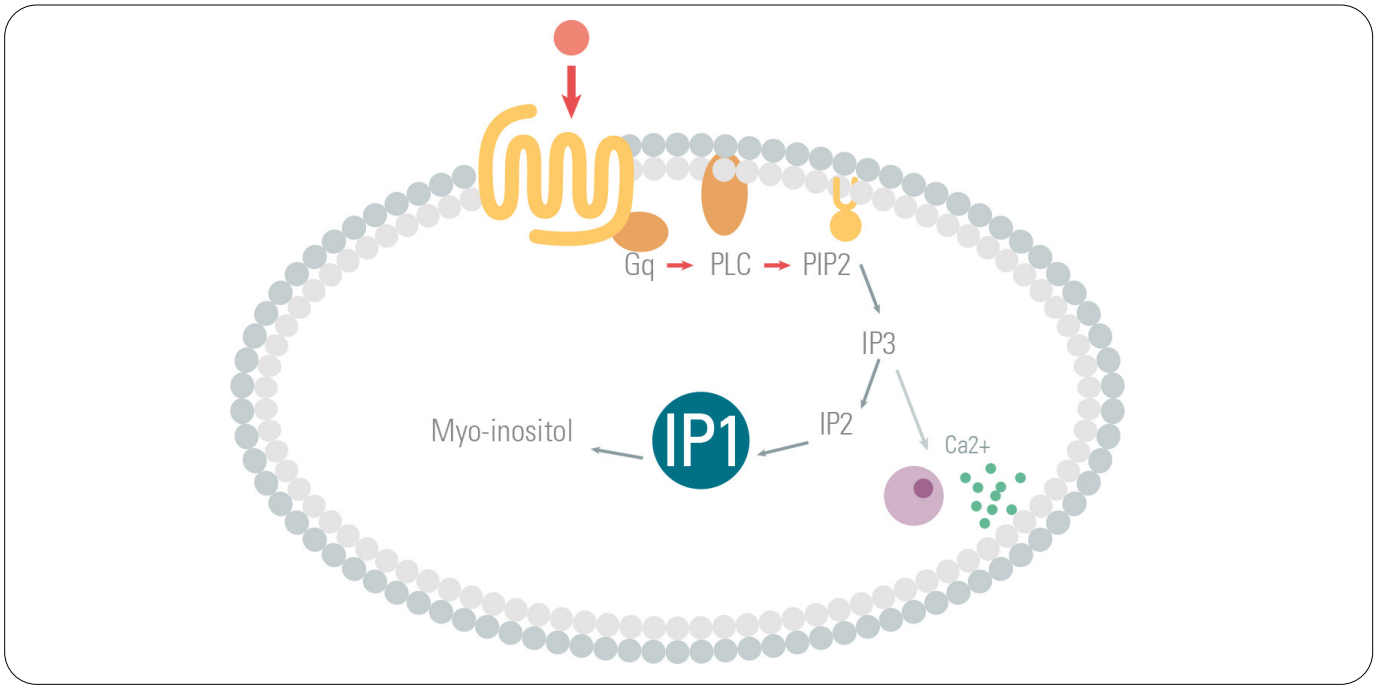

Figure 1: Cell signaling pathway illustrating Gαq-coupled GPCR receptor activation leading to the downstream production of IP1 and Ca++.

Materials and methods

Reagents & devices

- HTRF IP-One Tb assay, 20,000 tests (62IPAPEC)
- PHERAstar® FS reader (BMG Labtech)
- Greiner 384-well low volume white microplates (#784075)

IP-One assay protocols

Reagents were prepared and assays performed according to the instructions in the Product Insert with several experimental variables to generate stability data. These experiments were designed to reflect typical high throughput screening scenarios:

- Detection reagents [IP1-d2 conjugate (IP1-d2) and anti-IP1 cryptate Tb conjugate (Ab-K)] were reconstituted in the usual way to prepare stock solutions.
- Working solutions of IP1-d2 and Ab-K were prepared, aliquoted, and stored frozen (standard protocol = reference), at room temperature (RT), and at 4°C in HDPE (high density polyethylene) vials. (Time of preparation = t0)
- Standard curves were run using these conjugates at t0, t24, t48 and t96 hours. (Standards were freshly prepared in diluent buffer for each assay.) These assays were read on a PHERAstar® FS reader after 1 hr incubation at room temperature (standard protocol).
- In another experiment, a standard curve was run using freshly prepared reagents and the plate was read at the usual 1 hour and after 1–8 days' storage.
- In a final experiment, the IP1-d2 and Ab-K conjugates (working solutions) were premixed. Standard curves were run to compare results with premixed and separate conjugates.

Data Reduction

1. The HTRF Ratio was calculated for each well using the following formula:

$$\text{HTRF Ratio} = \frac{(\text{signal 665 nm})}{(\text{signal 620 nm})} \times 10^4$$

2. The Signal to Background (S/B) was determined as follows : S/B = HTRF Ratio max/HTRF Ratio min
3. S/B, EC50 and HTRF ratios were determined.

Results and discussion

Detection reagent stability: provides efficiency and consistency

Highly stable reagents provide significant benefits in a screening setting by allowing large batch preparation, in turn avoiding assay variability, reducing costly dead volumes, and eliminating daily preparation time.

This experiment demonstrates the stability of IP-One detection reagents (conjugates) over time at different storage temperatures by evaluating changes in HTRF ratio, S/B, and IC50. As shown in Fig. 2 and Table 1, results using conjugates stored at room temperature and 4°C are equivalent to those using frozen conjugates (reference). IP-d2 and Ab-K conjugates are very stable at room temperature and 4°C for up to 96 hours. IP-One detection reagents can be prepared and stored at these conditions, without any degradation or loss of assay performance.

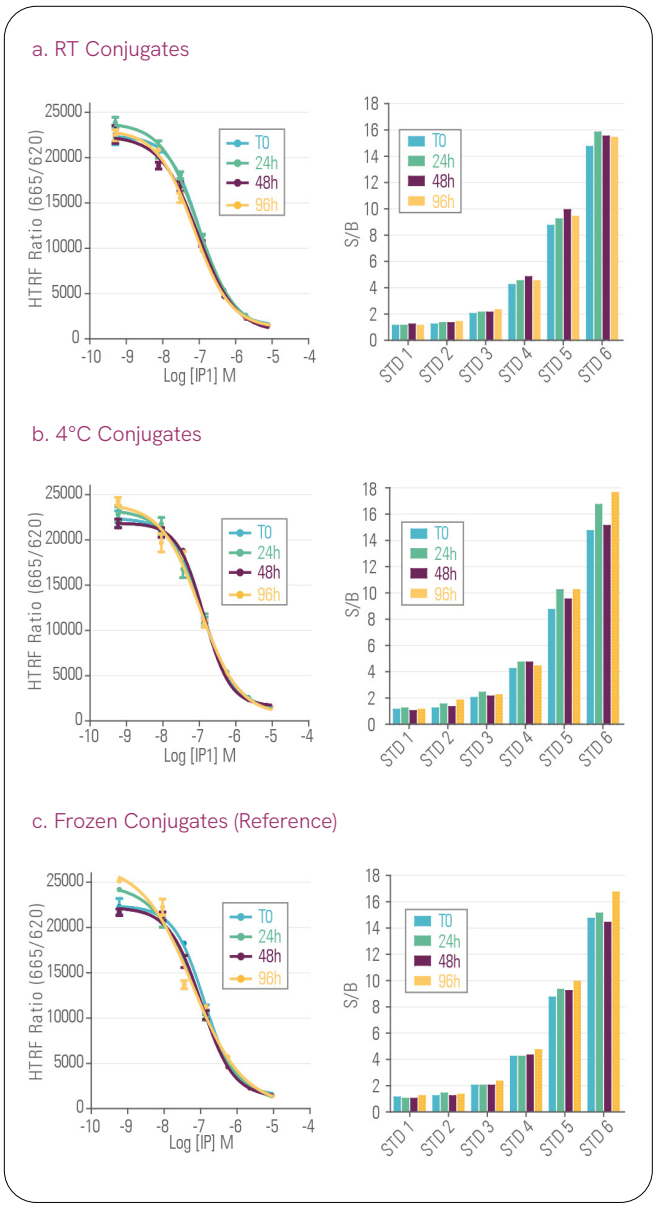

Figure 2: Stability of working solutions of detection reagents (IP1-d2 and Ab-K conjugates) is shown by comparing HTRF ratios and S/B over time after storage at a. RT, b. 4°C and c. frozen (reference). Small signal modulations after room temperature and 4°C storage are not significant (also seen in frozen reference).
Note: S/B STDx = HTRF Ratio STD0 (= Max Signal) / HTRF Ratio STDx

Table 1: IC50 values (M) for standard curves in Fig. 2 generated with conjugates stored at a. RT, b. 4°C and c. frozen (reference) after fresh preparation and storage for 24, 48 and 96 hours. Small IC50 modulations after room temperature and 4°C storage are not significant (also seen in frozen reference).

Storage Temp	RT	4°C	Frozen (Reference)
T0	1.1e-07	1.1e-07	1.1e-07
T24	9.5e-08	9.6e-08	6.6e-08
T48	9.2e-08	1.1e-07	8.8e-08
T96	7.2e-08	9.0e-08	5.1e-08

Signal stability: allows plate storage and later read

For an assay to be suitable for HTS, a highly stable assay signal is mandatory. If the signal is unstable, plates must be read shortly after reagent additions, potentially limiting throughput. Furthermore, any interruptions in the screening operation will result in signal decay and eventual loss if not addressed quickly.

This experiment demonstrates the stability of the IP-One signal and IC50 over time. As shown in Fig. 2 and table 2, the variability of the IC50 calibration curve was remarkably lower than 10% when plates were left at room temperature for eight consecutive days. The assay window remained high at 12.8 compared to initial value of 14.7 fold. Plates can be stored at room temperature and read at a later date, days after the dispensing, without the information being lost. The strong signal stability also protects the data if delays in the screening operation are encountered during the working day.

With the IP-One assay, throughput is not limited by signal stability. Use of laboratory resources can be maximized because results can be read at any point in time.

Table 2: IC50 values (M) and assay window for standard curves in Fig. 3 generated with a plate read at the usual 1 hour and after 1 – 8 days' storage.

Incubation time	IC50	Assay window
1h	1.07e-07	14.7
1 day	1.07e-07	14.6
2 days	1.19e-07	13.1
5 days	1.13e-07	13.3
8 days	1.14e-07	12.8

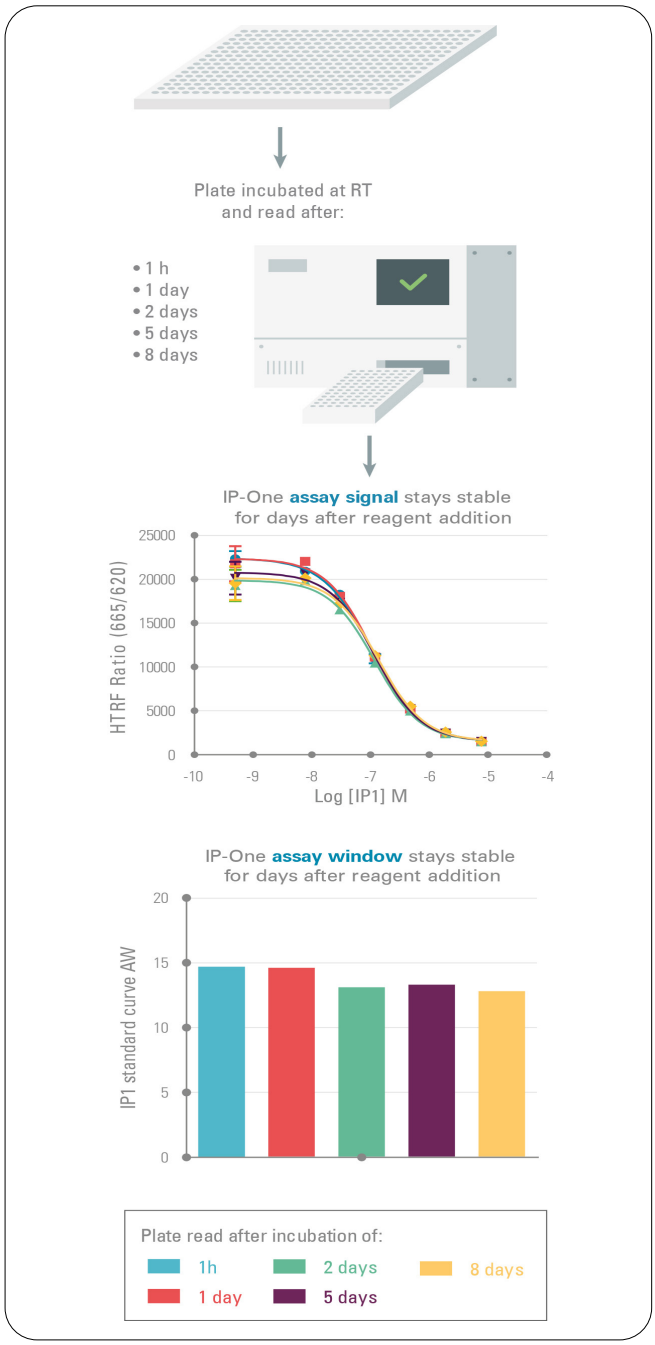

Figure 3: The IP-One signal is very stable for at least eight days following addition of the detection reagents.

Premixed conjugates: Maximizes ease-of-use, throughput and consistency

Implementing the IP-One assay on an HTS platform is very simple and already requires limited steps. Additional time efficiency and throughput can be gained by premixing the IP1-d2 and Anti-IP1 cryptate Tb (Ab-K) conjugates.

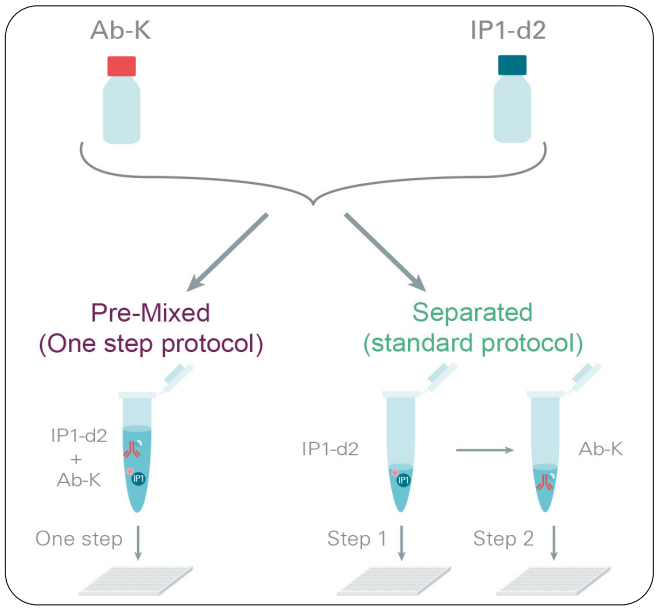

Figure 4: Standard vs. streamlined assay protocol with premixed conjugates.

In this experiment, two standard curves were run, one using the standard two-step protocol (successively dispensing IP-d2 and Ab-K) and one using premixed conjugates dispensed in one step (IP-d2 mixing conjugates streamlines the assay process without compromising results. This saves time, and reduces dead volume and variability, especially beneficial in large screening runs.

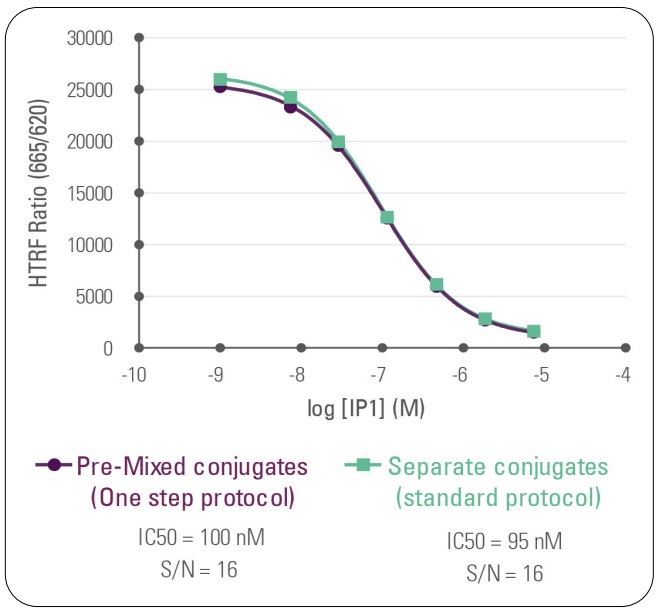

Figure 5: IP-One assay with premixed and separate conjugates results in essentially identical standard curves.

Conclusion

Revvity HTRF IP-One kit is a robust, easy-to-use assay designed to fit the unique needs of high throughput screening environments. Labs can prepare large batches of detection reagents and store them ready to use conserving valuable materials, time and resources. The assay's long-lasting signal, stable at least eight days, allows for delayed plate reading, not possible with calcium assays, without compromising results or losing data. Additionally, with the IP-One assay, throughput is not limited by signal stability because results can be read at any point in time. In conclusion, the HTRF IP-One kit is an ideal choice for HTS campaigns requiring downstream measurement of Gαq-coupled GPCR receptor activation.

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