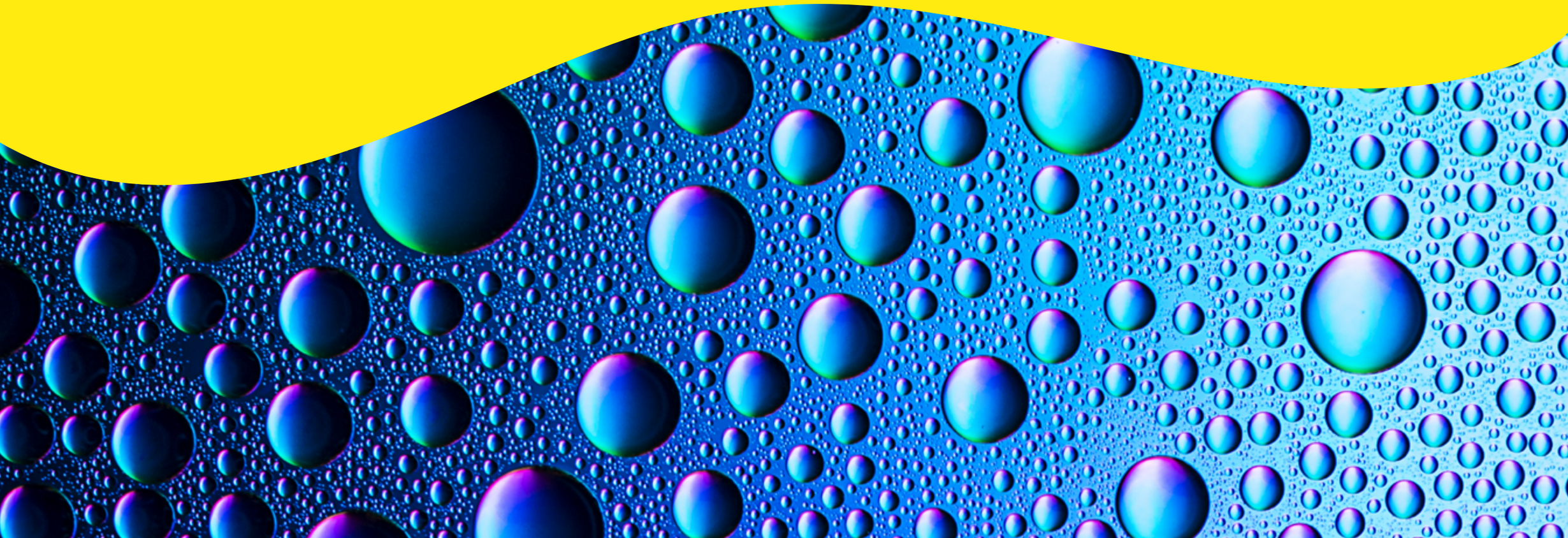
IP-One

Guide to what every GPCR scientist should know about IP-One.



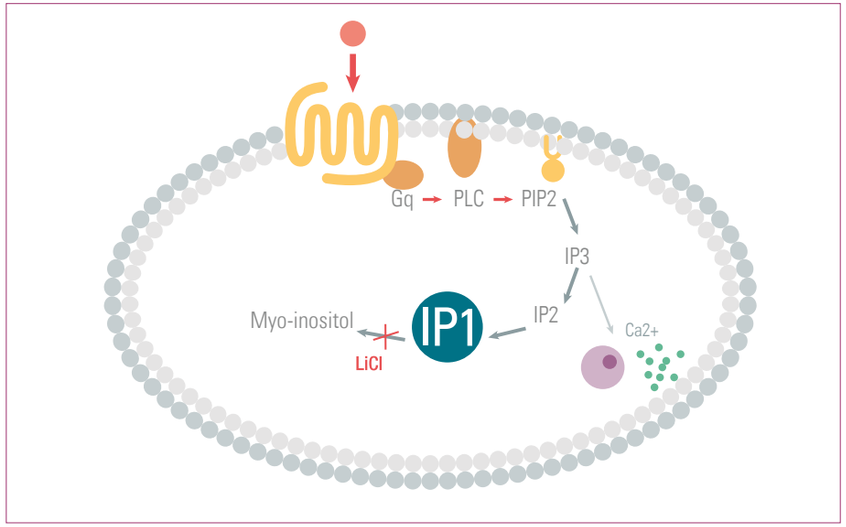
OVERVIEW

Signaling Pathway

Revvity offers an innovative approach with IP-One, a non-radioactive, homogeneous, inositol-phosphate accumulation assay for Gαq signaling. This kit has been shown to offer superior benefits over traditional assays, making it the ideal tool for your research.

Revvity's IP-ONE is a reliable and recognized tool for investigating the Gq/11 coupled receptor.

Revvity has developed a **GPCR platform**, based on the company's HTRF® technology, to measure second messenger accumulation. This platform includes IP-One, a very unique, non-isotopic assay. This bioassay kit specifically measures IP1 levels in cells. Over the past decade, IP-One has demonstrated its efficacy on numerous occasions for discovering and/or characterizing new compounds that target GPCRs.



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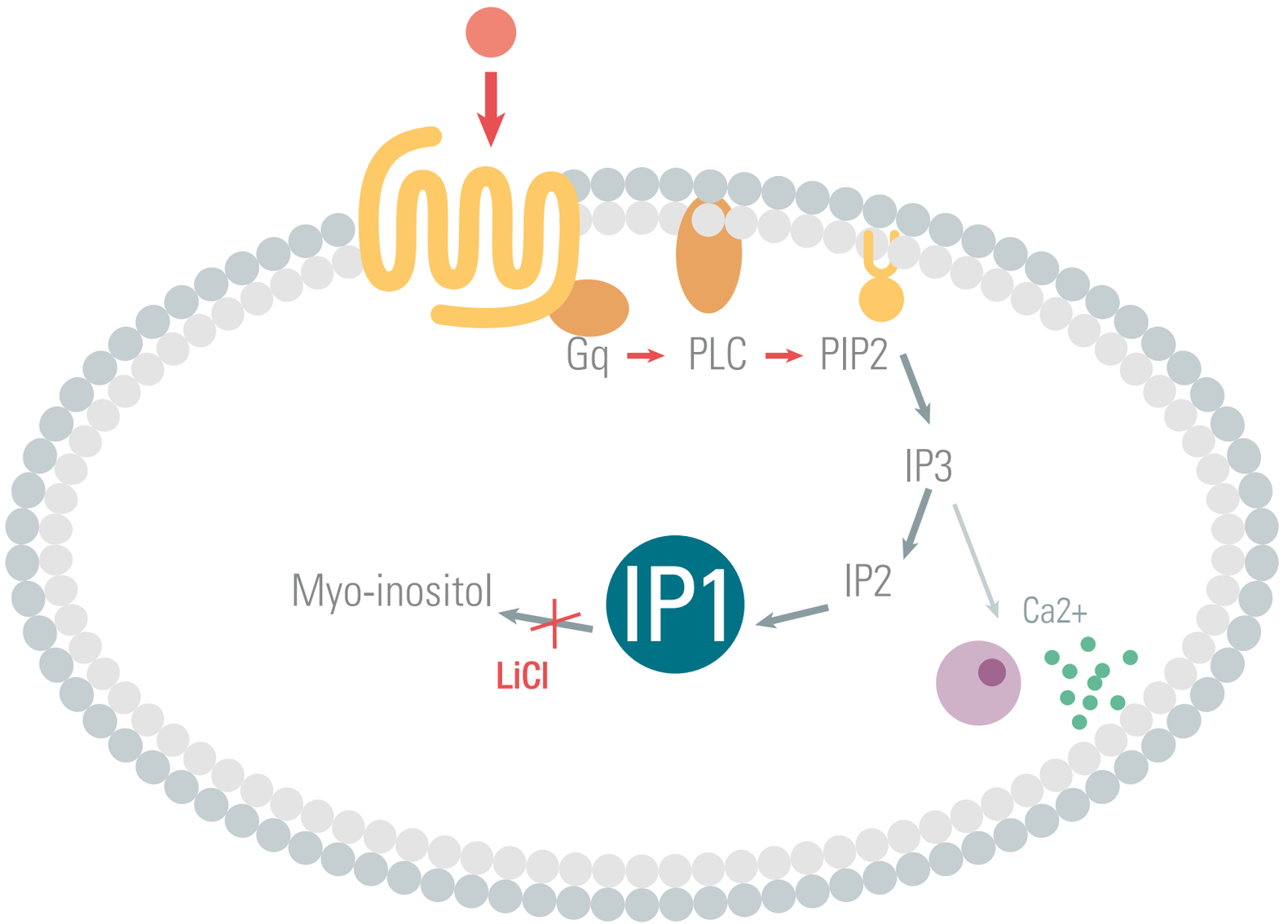

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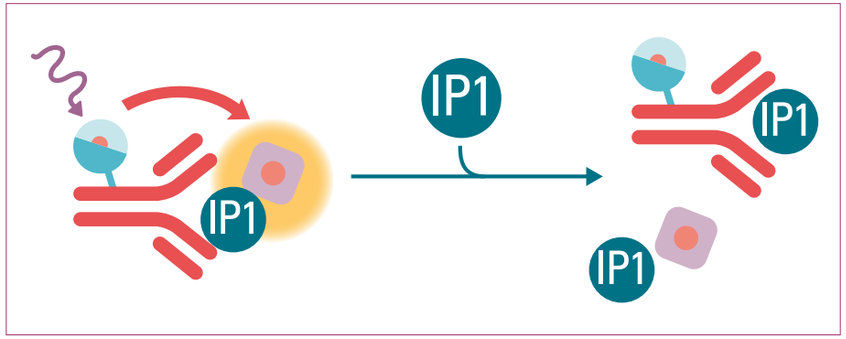
Assay Format

IP-One is a patented competitive immunoassay involving a specific antibody terbium cryptate (donor) and IP1 coupled to D2 (acceptor).

In a standard state, the interaction between the donor and the acceptor produces a fluorescent signal. When unlabeled IP1 is present in the sample (due to GPCR activation), binding competitively to the antibody and taking the place of the labeled IP1. Fluorescence then decreases, as the donor is no longer in close proximity to the acceptor. The range of the decrease is directly related to IP1 accumulation.



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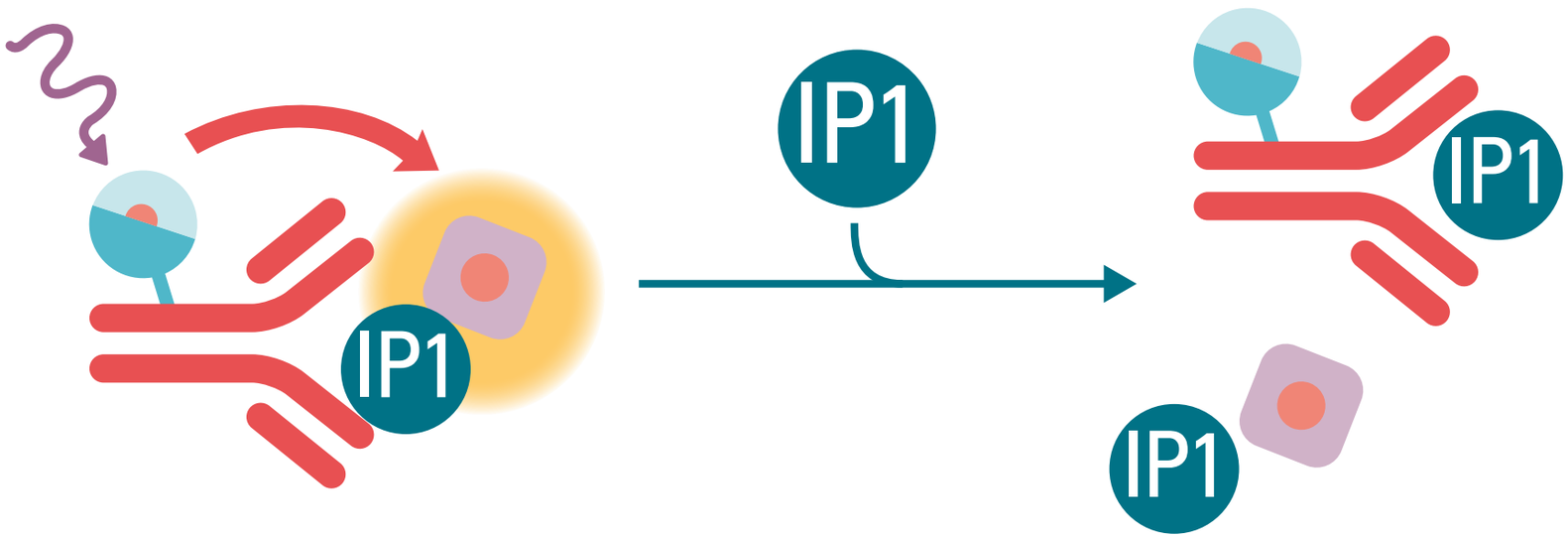

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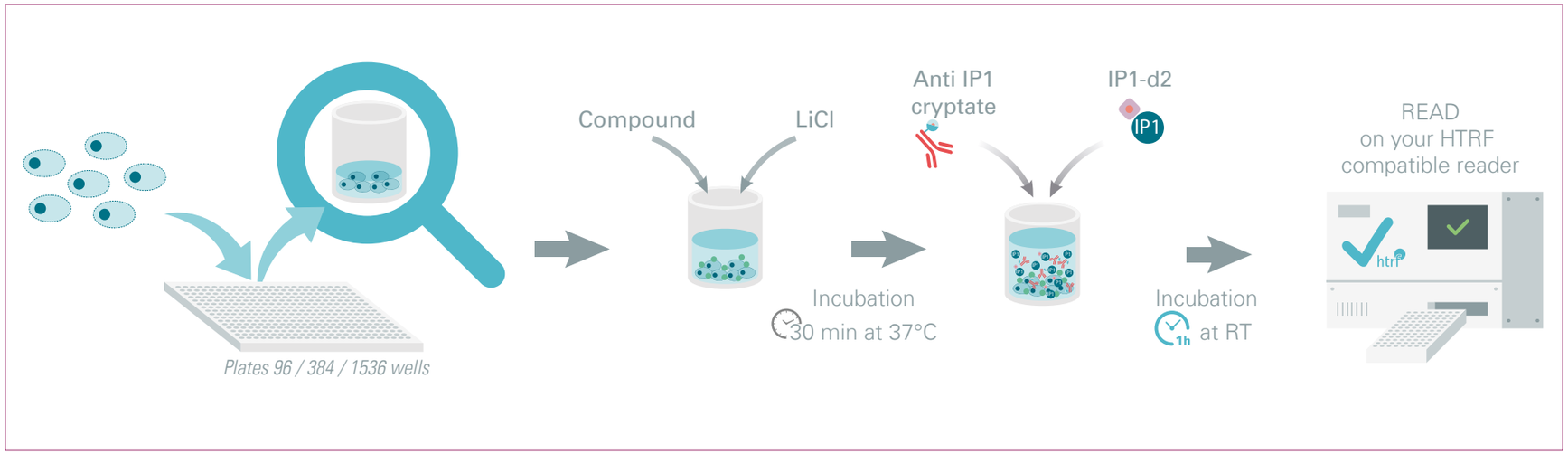
Assay Protocol

As with most HTRF-based assays, IP-One is very easy to set up. The IP-One assay protocol involves two incubation steps:

- Cell stimulation by the ligand or target compounds
- IP1 detection using HTRF reagents

The kit provides everything you need, including the Lithium Chloride used to prevent IP1 degradation.



This protocol requires **only a single, one-hour incubation period** following cell stimulation.

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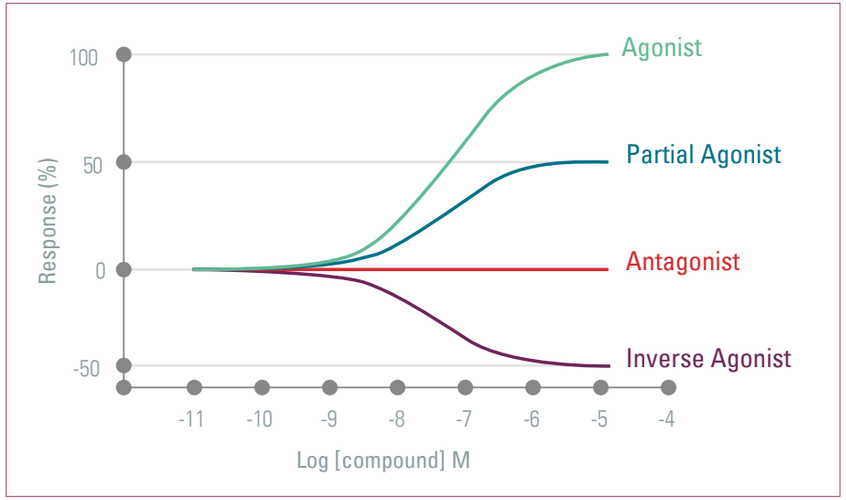
Pharmacology

Ip-one enables investigation of all classes of pharmaceutical compounds

IP-One makes it possible to assess all classes of pharmaceutical compounds, namely:

- Agonists
- Antagonists
- Allosteric modulators
- Inverse agonists
- Slow-acting binders



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Benefits

Choosing IP-One for investigating Gq/11 coupled receptors offers **numerous benefits:**

- Simple protocol and assay kit
- Miniaturization down to 1536-well plates
- Receptor proximal marker
- Compatible with primary and non-recombinant cell lines
- And more…

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Packaging

To better answer all your needs, IP-One is available in different technologies: Alpha and HTRF and varying sizes.

We are also able to deliver the exact volume of reagent, in a single batch, needed to match your screening requirements.

HTRF IP-One Gq	
Kit size	**Part #**
1,000 assay points	62IPAPEB
10,000 assay points	62IPAPEC
100,000 assay points	62IPAPEJ

AlphaLISA IP-One	
Kit size	**Part #**
1,000 assay points	AL3145D
10,000 assay points	AL3145M
50,000 assay points	AL3145R

There will always be a **solution adapted to your needs.**

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HIGH THROUGHPUT SCREENING

Ease-of-use

When imagining an assay that is HTS compatible, the first thing that most people think about is performance and miniaturization. Of course, IP-One answers these needs, but there is more. Reagent stability and signal stability may seem less critical at first glance, but they are both highly necessary for successful screening.

This section provides an in-depth focus on the points that make IP-One a true, trusted assay for HTS.

IP-One: a homogeneous assay

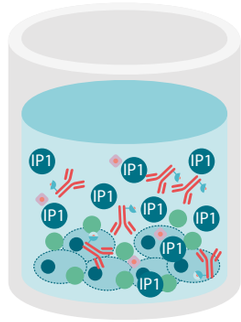
As mentioned earlier, IP-One is a very simple assay to use. Some suppliers' assays may be limited with respect to HTS requirements, but not the HTRF assays on which IP-One is based!

Homogeneous "**add-and-read**" assays are well-suited for HTS, as they avoid filtration, separation, and wash steps that can be both time-consuming and difficult to automate.

Using IP-One for your screen offers several **advantages**:

- No complex robotic plate washers required
- Eliminates equipment maintenance costs
- Saves you time by minimizing the number of steps needed to run the assay

ADD and
READ



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HIGH THROUGHPUT SCREENING

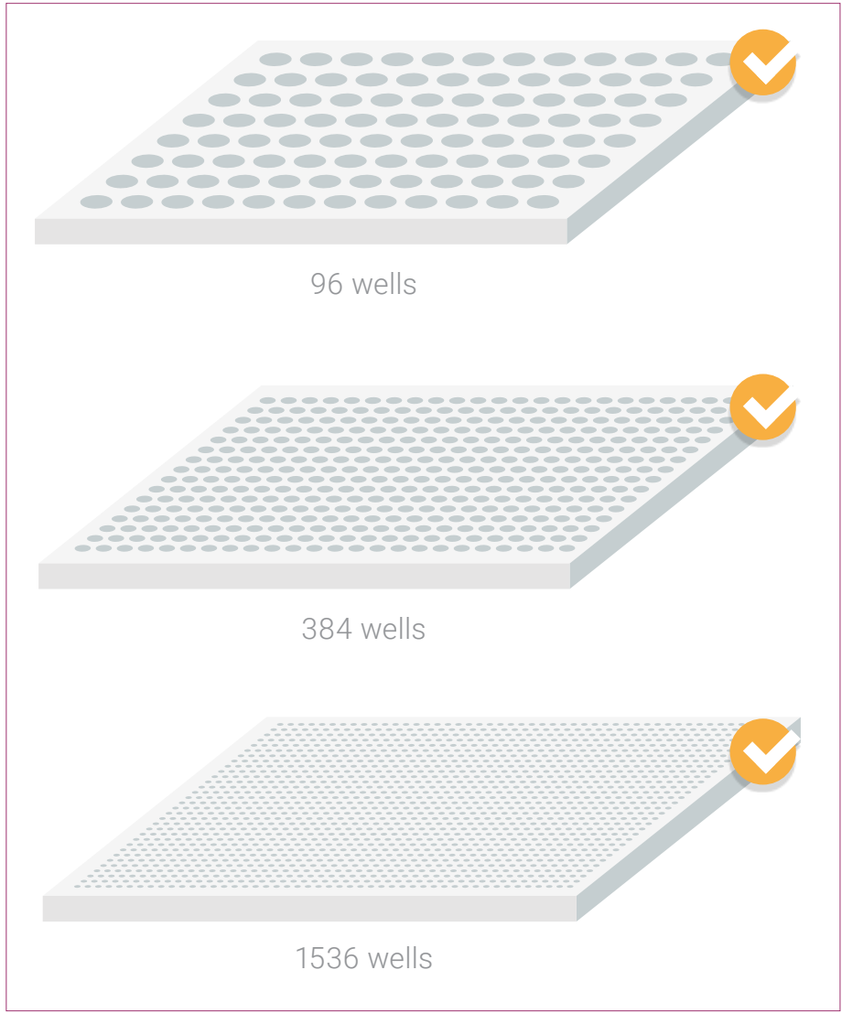
Ease-of-use

Miniaturization

Miniaturization lowers reagent consumption and increases throughput. Revvity considers that an assay **must adapt to your needs**, not the other way around!

IP-One can be implemented from 96w (100 µL or 20 µL using our new HTRF 96-well low volume plate) to 384w or even 1536w plates (10 µL), depending on your specific situation.

Revvity always has **the right solution** for you.



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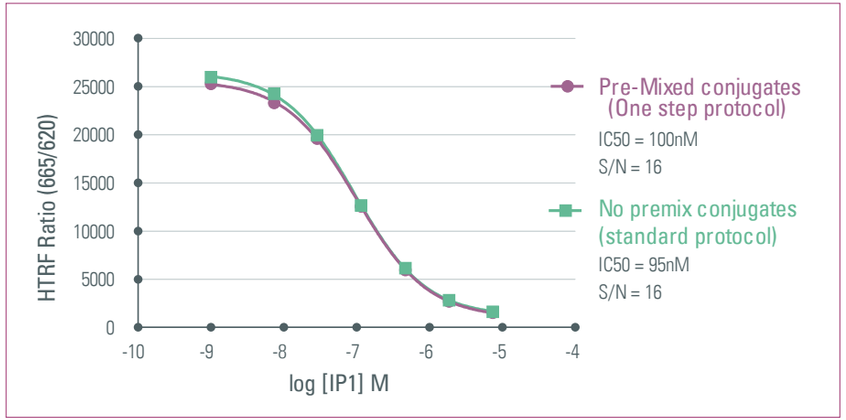
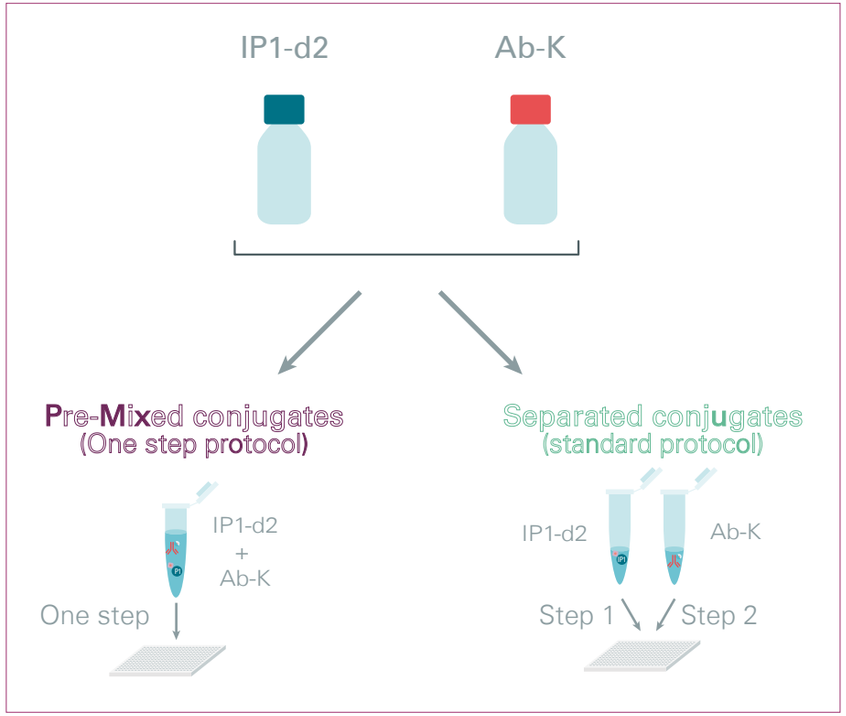
Ease-of-use

IP-One can make it even easier

The classic protocol for IP-One requires preparation of two working solutions (one for IP1-d2, the other for Ab-K), followed by two successive dispensing operations.

Here, we demonstrate the possibility of premixing your conjugates, which makes it easier to use IP-One, and streamlining the process without compromising your results.





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HIGH THROUGHPUT SCREENING

Ease-of-use

IP-ONE: no expensive plate reader required.

While an intracellular calcium assay requires the use of a costly kinetic fluorescence assay, the IP-one assay can be performed using a **standard fluorescence plate reader.**

More than 50 certified or compatible instruments, and counting! Your lab might even already have one. If not, our scientific team will help you choose the instrument that best suits your needs and budget.

Revvity offers two microplate readers certified for use with HTRF: Envision® and VICTOR® Nivo™.

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HIGH THROUGHPUT SCREENING

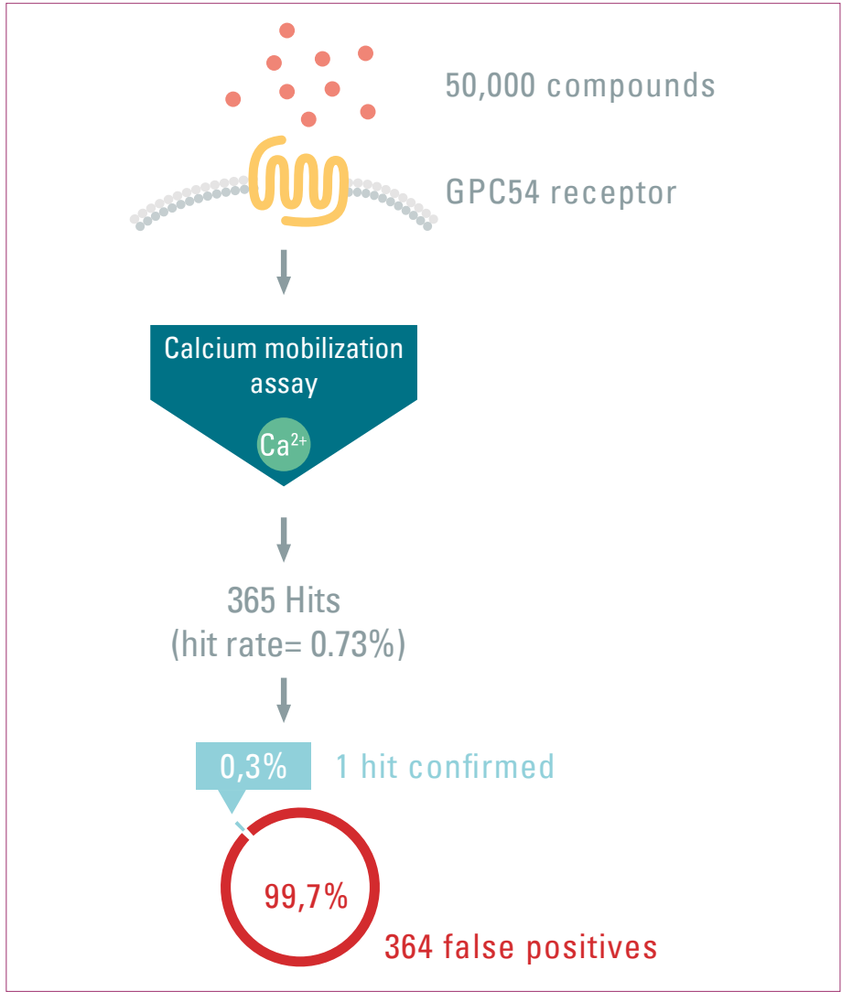
Performance

Fewer false positives: IP-ONE brings more specificity to your screens

There is no need to demonstrate how false positives can have a negative effect on your screen: they lead to extra work and incur additional cost, as the false positives need to be eliminated.

IP-One simply delivers fewer false positives than calcium flux technologies, practically eliminating the need to counter-screen the hits obtained from primary screening.

In this example, representative of a typical workflow with a calcium flux screen, 50,000 compounds were screened and 365 hits were obtained. In reality, 99.7% of the hits were false positives. Only one hit was confirmed with multiple technologies (including IP-One).



This workflow was adapted from: K J. Cassutt *et al* «Identifying Nonselective Hits from a Homogeneous Calcium Assay Screen,» Journal of Biomolecular Screening 12(2) (2007)

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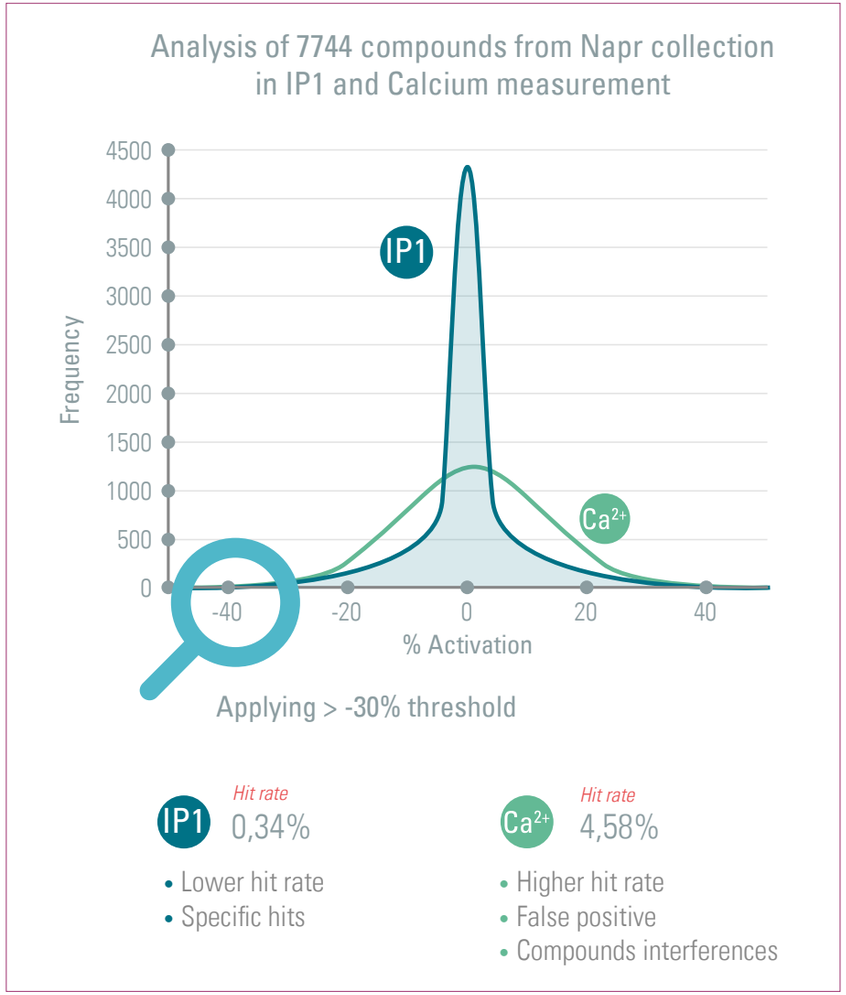
Performance

The high false positive rate associated with calcium flux technologies leads to an erroneous assessment of the discovery rate, resulting in an inflated distribution of hits.

IP-One reduces the false positive rate, displaying a lower hit rate but with almost only specific hits.



Adapted from: Rochdi Bouhelal presentation at SBS 2005 – Novartis Institutes for BioMedical Research.

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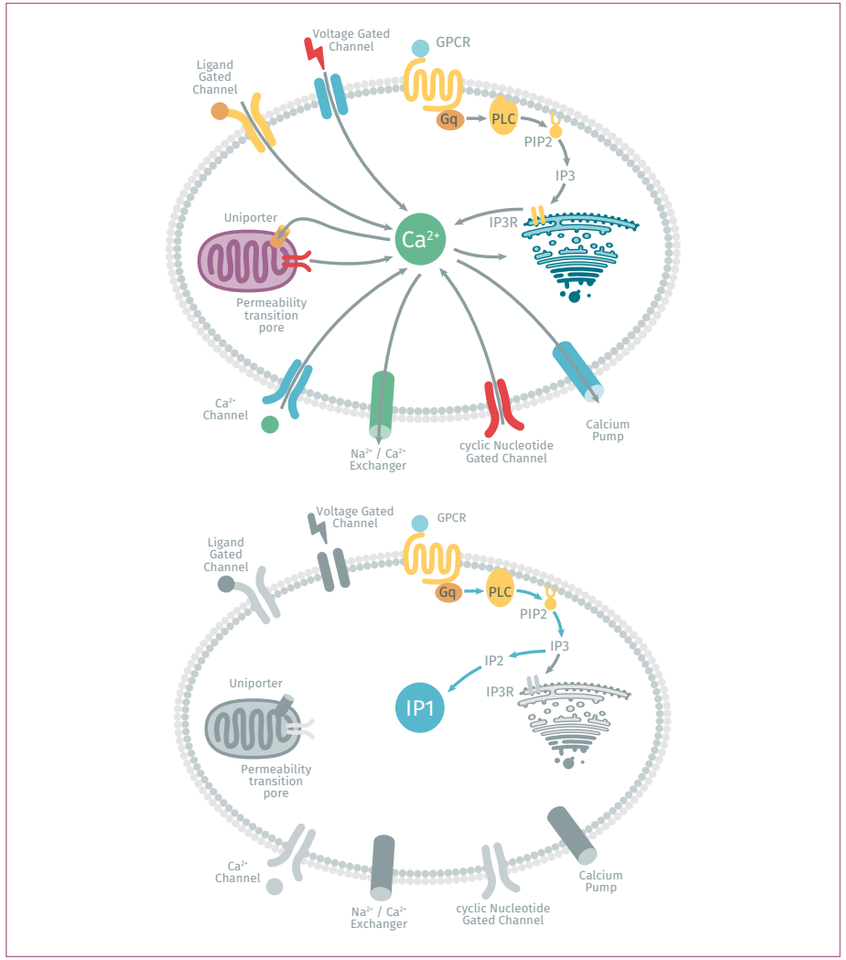
Performance

Numerous of reasons can be found to explain the high rate of false positives obtained with calcium flux assays. Calcium responses are complex in nature. Calcium release is frequently triggered by non-G protein mechanisms (Ca^{2+} permeable channels, Ca^{2+} pumps, and Ca^{2+} transporters). Compounds engaging these receptors are said to be “off-target”.

IP-One **ensures signal specificity** reducing the high rate of false positives.

It only measures IP1, produced solely by phospholipase enzyme (PLC) action and mediated by GPCRs.

Using calcium flux technology, Ca^{2+} ionophores and compounds that permeabilize the cellular membrane appear as false positives in agonist screening assays. IP-One ensures that these compounds remain undetected.



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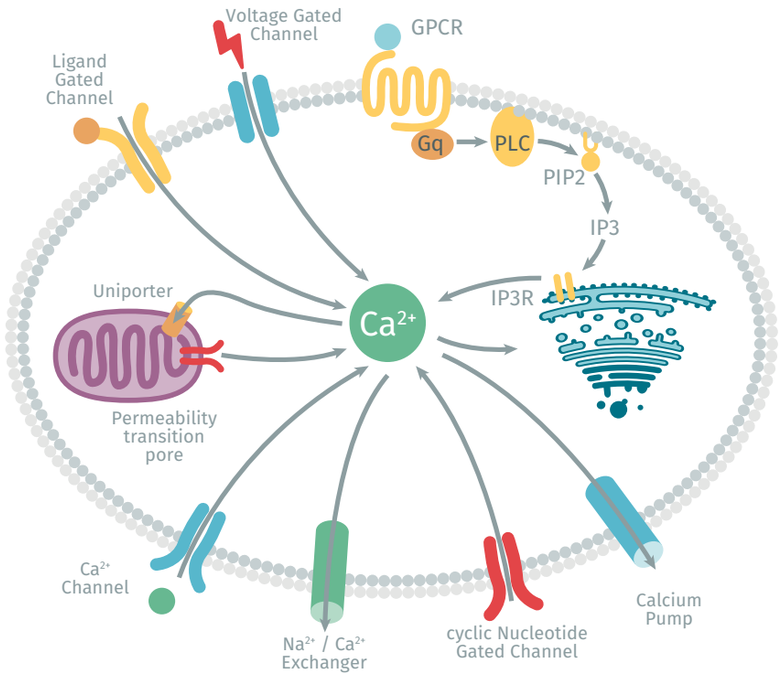

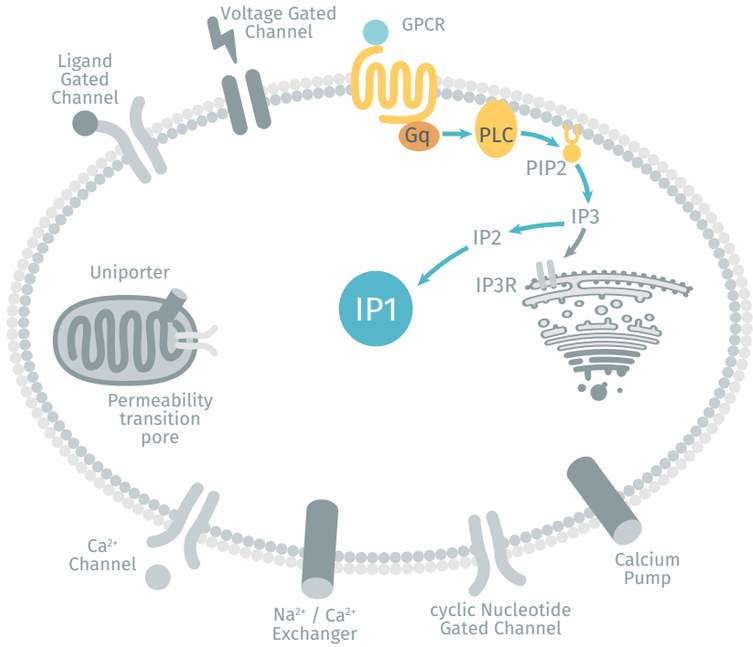

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HIGH THROUGHPUT SCREENING

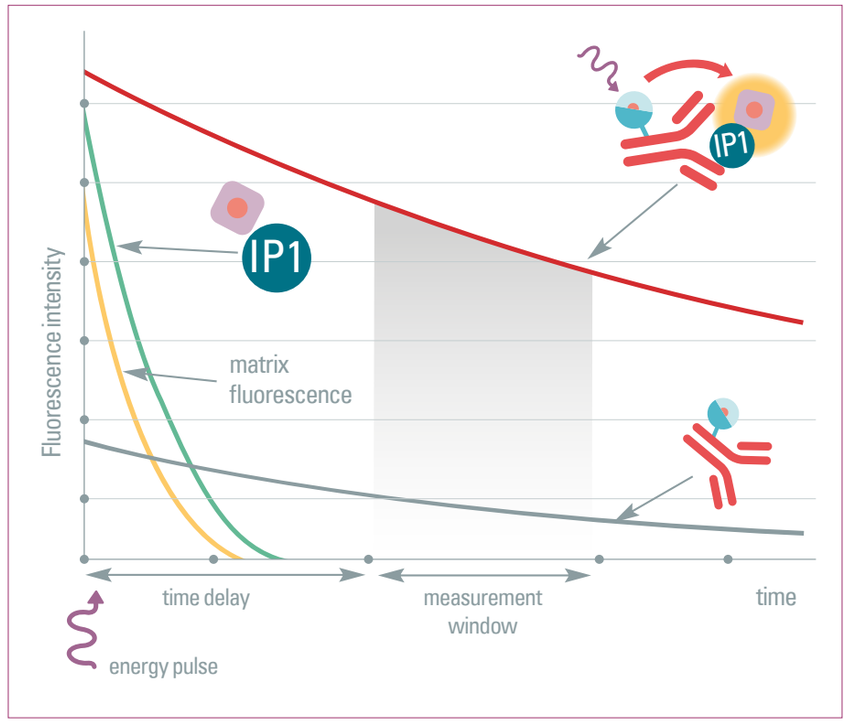
Performance

Using calcium flux assays, you may be confronted with:

- Blocked tips that lead to false positives in inhibitor screens, or false negatives in agonist screens.
- Auto-fluorescent compounds that appear as false positives in agonist mode.

Standard compound libraries used for a screen may contain a significant number of auto-fluorescent compounds. Based on a time-resolved signal, IP-One eliminates the emission signal from most auto-fluorescent compounds automatically, thus lowering the rate of false positives.

The energy pulse from the excitation source (flash lamp or laser) is immediately followed by a time delay, allowing the decay of interfering short-lived fluorescence, such as from compounds, proteins, or medium.



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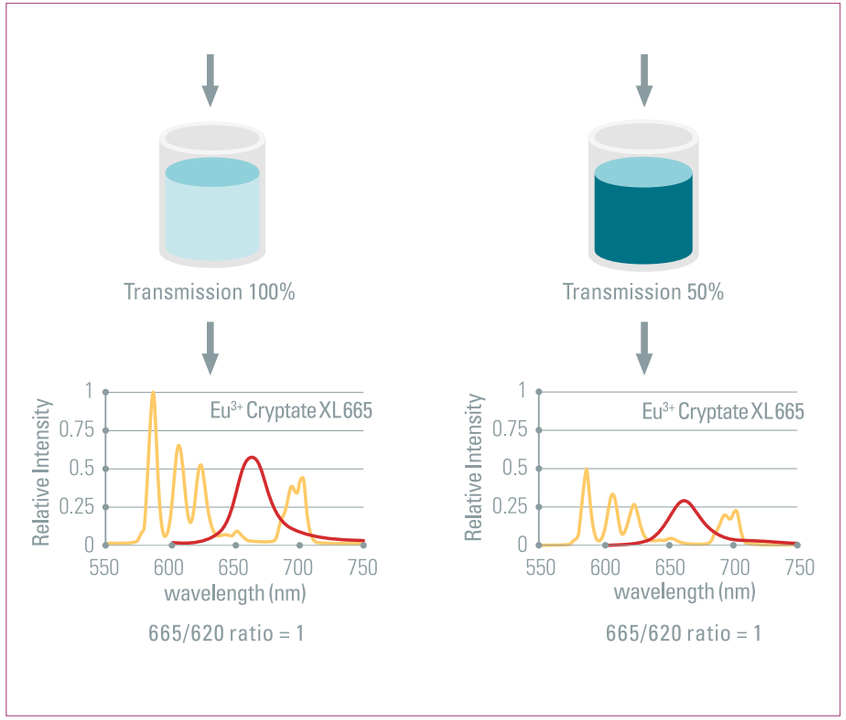
Performance

Another advantage of HTRF is that it uses a specific ratio, which protects results from any inner filter effects. HTRF emissions are measured at two wavelengths: **620nm (donor)** and **665nm (acceptor)**.

Dual-wavelength measurements are extremely advantageous and help compensate for well-to-well variations. Filter effects may occur when highly colored compounds are used, leading to a decrease in the excitation energy delivered. The fluorescence ratio **compensates for this phenomenon** because both acceptor and donor emissions are affected in the same manner.



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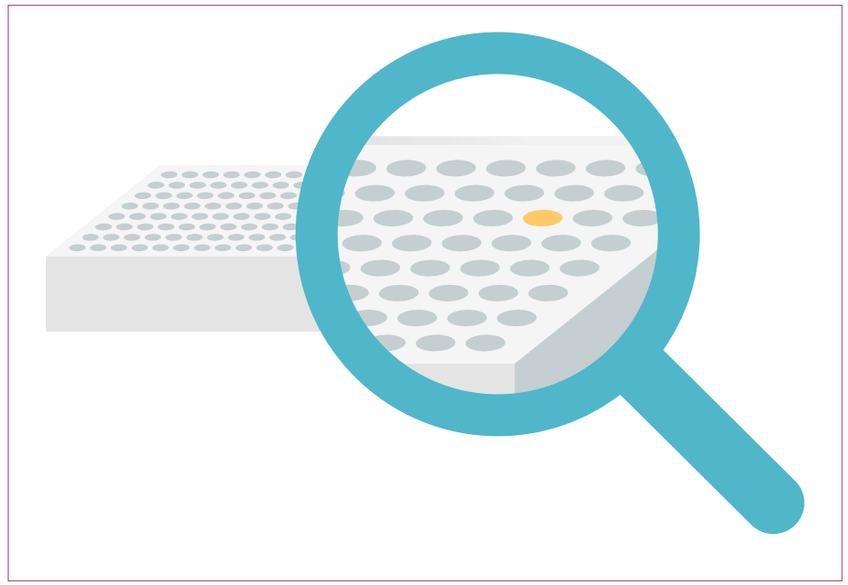
HIGH THROUGHPUT SCREENING

Performance

Low false negative rate: don’t miss your hits anymore

Having a high false positive rate may cost you time and money, but your hit is still there in the end. On the other hand, a high false negative rate can have disastrous impact on your screen. What if the molecule you were looking for was really there, your future block-buster, but you missed it because it was undetected by your screening technology?



The consequences of **false negatives** are **too serious** to tolerate.

True negative or missed hit?

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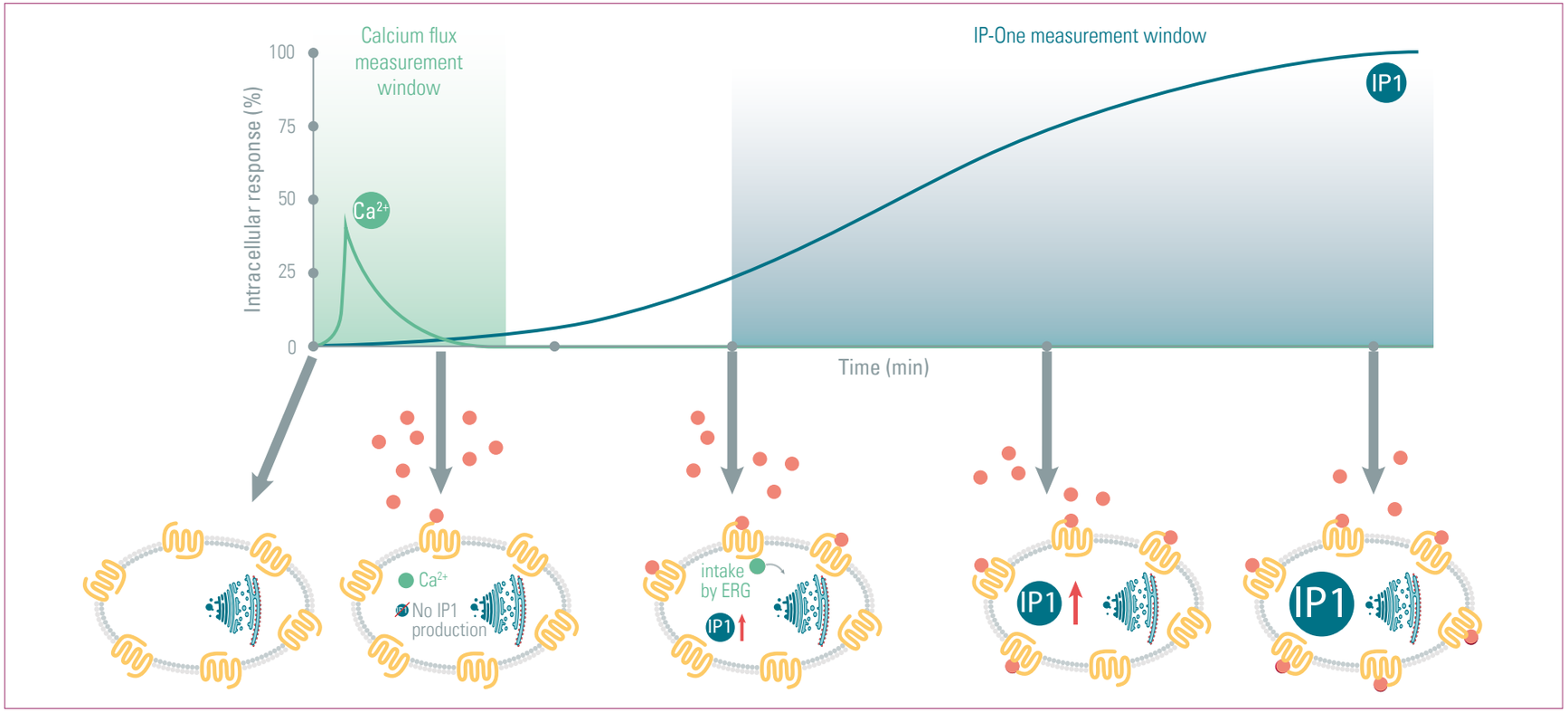
Performance

Interpretations of calcium assay results may be hindered because of the non-equilibrium state. The absence of equilibration results in biased IC50 for inhibitors, and false negatives in screening campaigns in which important molecules may be lost forever.

IP-One is built on an accumulation assay and allows you to record every possible hit, including slow-acting compounds with true pharmacology associated with accurate IC50 determination.



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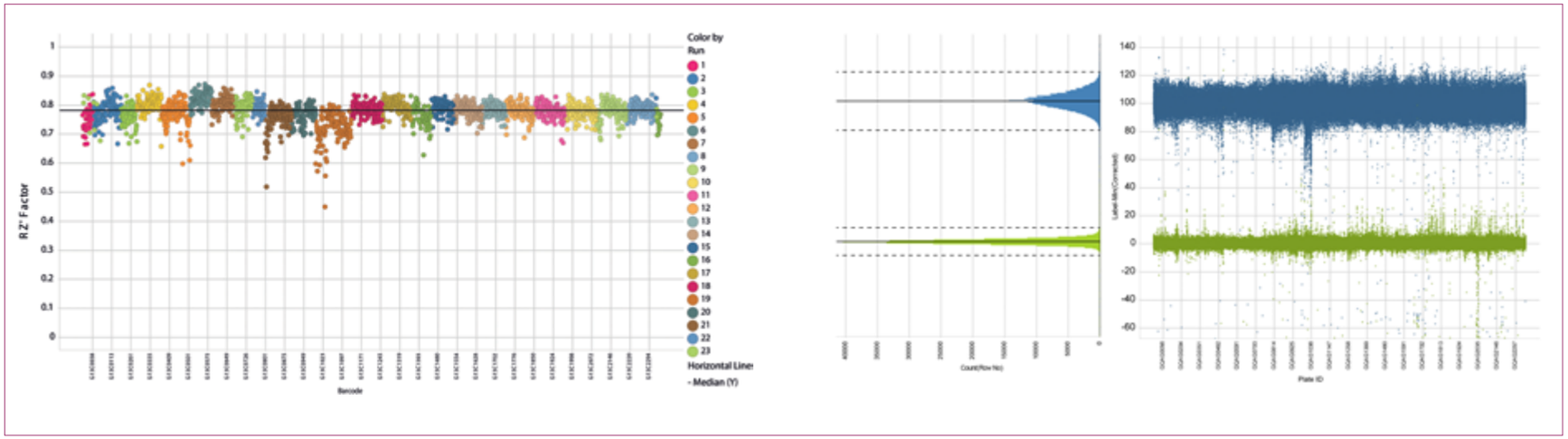
HIGH THROUGHPUT SCREENING

Performance

Robustness: IP-ONE delivers high quality Z’ and high reproducibility

The Z’ score is perhaps the most widely-used statistical parameter for measuring assay robustness for HTS. In one case study by Bayer, IP-One screen proved excellent assay quality providing high robust Z’ factors in every run (~ 0.78) and broad separation of controls.

Here are some examples of data scatters obtained by Bayer in one case study:



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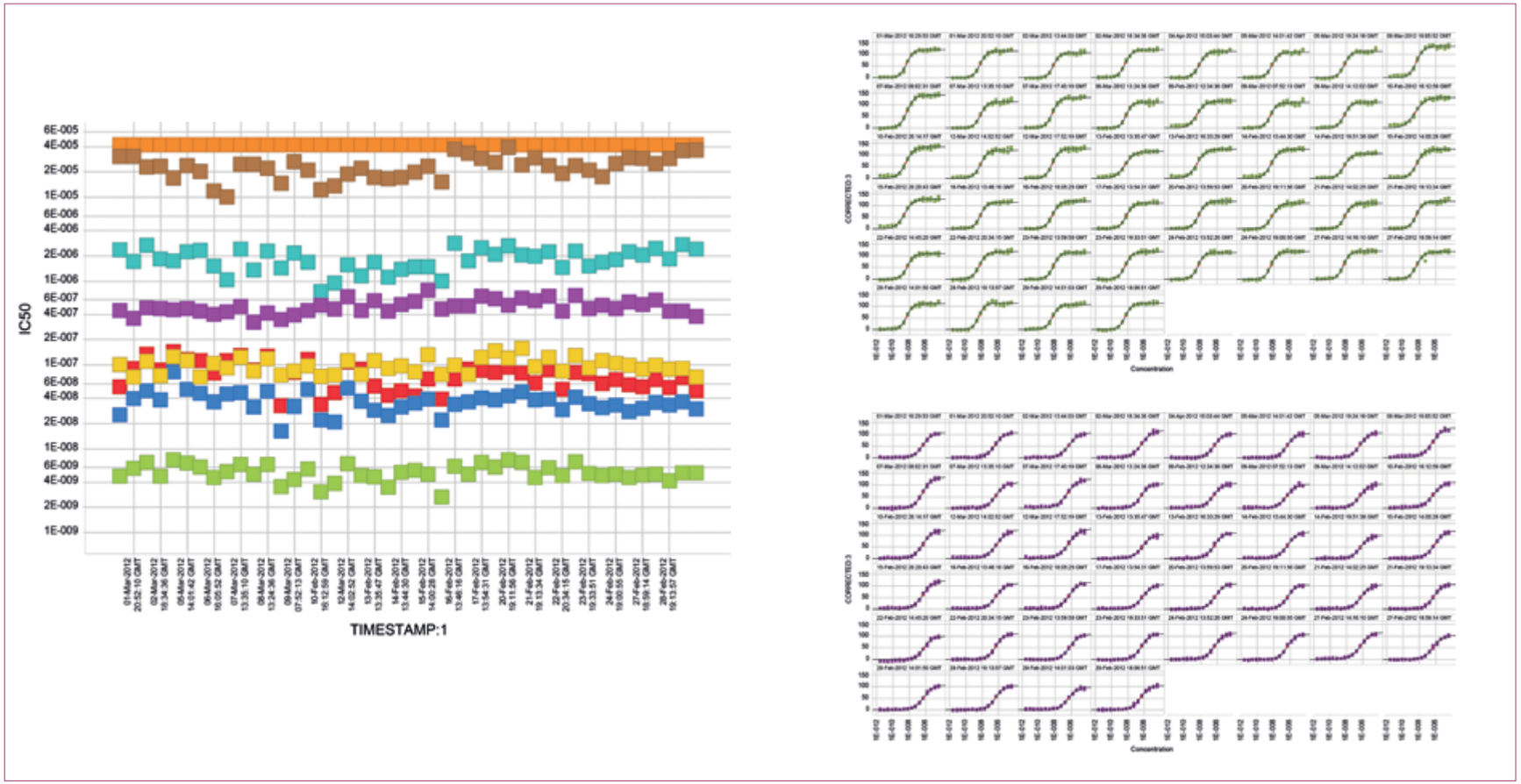
Performance

Bayer also obtained constant IC50 values for reference compounds over the entire screening in one case.



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Performance

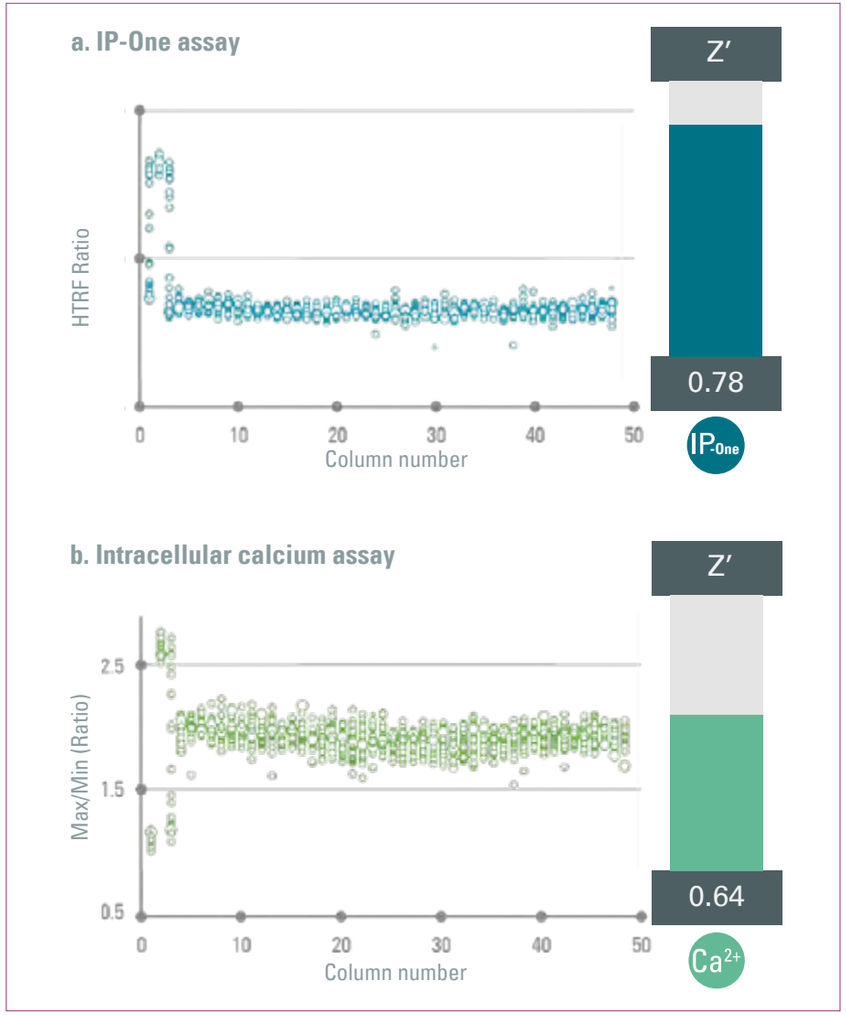
Robustness: IP-ONE Z' versus calcium assay Z'

By measuring Z' factor reliably, **IP-One stands out as a very robust assay.**

Several reasons can explain the differences in robustness between these two assays. For example, conventional calcium mobilization protocols are multi-step procedures, and each additional step carries the risk of increased assay variability.

This inevitably decreases Z':

- **Removal of cells** from wells during the wash procedure to purge excess dye
- **Reduced responsiveness** (competence) of cells after washing due to perturbation
- **Spontaneous calcium flux** upon the addition of buffer or compound
- **Incomplete washing** resulting in a significant signal drop upon the addition of test compound
- **Asymmetric release of probenecid** by cells



Adapted from: Liu K (2008), Curr Chem Genomics. Comparison on Functional Assays for Gq-Coupled GPCRs by Measuring Inositol Monophospate-1 and Intracellular Calcium in 1536-Well Plate Format.

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HIGH THROUGHPUT SCREENING

Performance

Robustness: Literature

A significant body of literature has demonstrated how **IP-One robustness outperforms calcium flux technologies.**

Some significant examples are listed below.

- *Bergsdorf* «A One-Day, Dispense-Only IP-One HTRF Assay for High-Throughput Screening of Gq Protein-Coupled Receptors: Towards Cells as Reagents.», Assay Drug Dev Technol (2008).
- *Liu K* «Comparison on Functional Assays for Gq-Coupled GPCRs by Measuring Inositol Monophospate-1 and Intracellular Calcium in 1536-Well Plate Format.», Current Chemical Genomics (2008).
- *Cassutt* «Identifying Nonselective Hits from a Homogeneous Calcium Assay Screen.», Journal of Biomolecular Screening (2007).
- *Kuohung* «A high-throughput small-molecule ligand screen targeted to agonists and antagonists of the G-protein-coupled receptor GPR54.», Journal of Biomolecular Screening (2010).
- *Thomsen* «Functional assays for screening GPCR targets.», Current Opinion in Biotechnology (2005).

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HIGH THROUGHPUT SCREENING

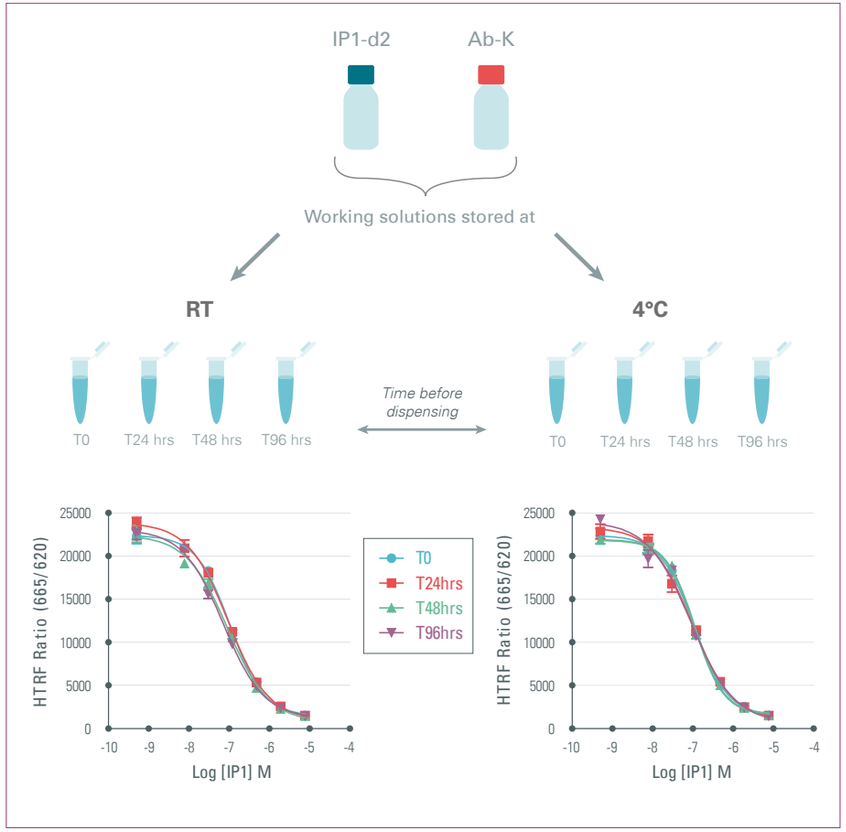
Performance

Stability: Revvity guarantees its reagent stability

When you screen thousands of compounds, it may take several hours before the reagents are added to all of the assay plates. Revvity reagent stability during and beyond daily screening operations (i.e. 8 hour shifts) has been confirmed.

Reflecting a typical screening campaign, the following study describes a series of stability tests conducted to confirm that IP-One reagents are stable:

- IP-One reagents are stable for several days at 4°C and room temperature
- IP-One delivers consistent performance over time



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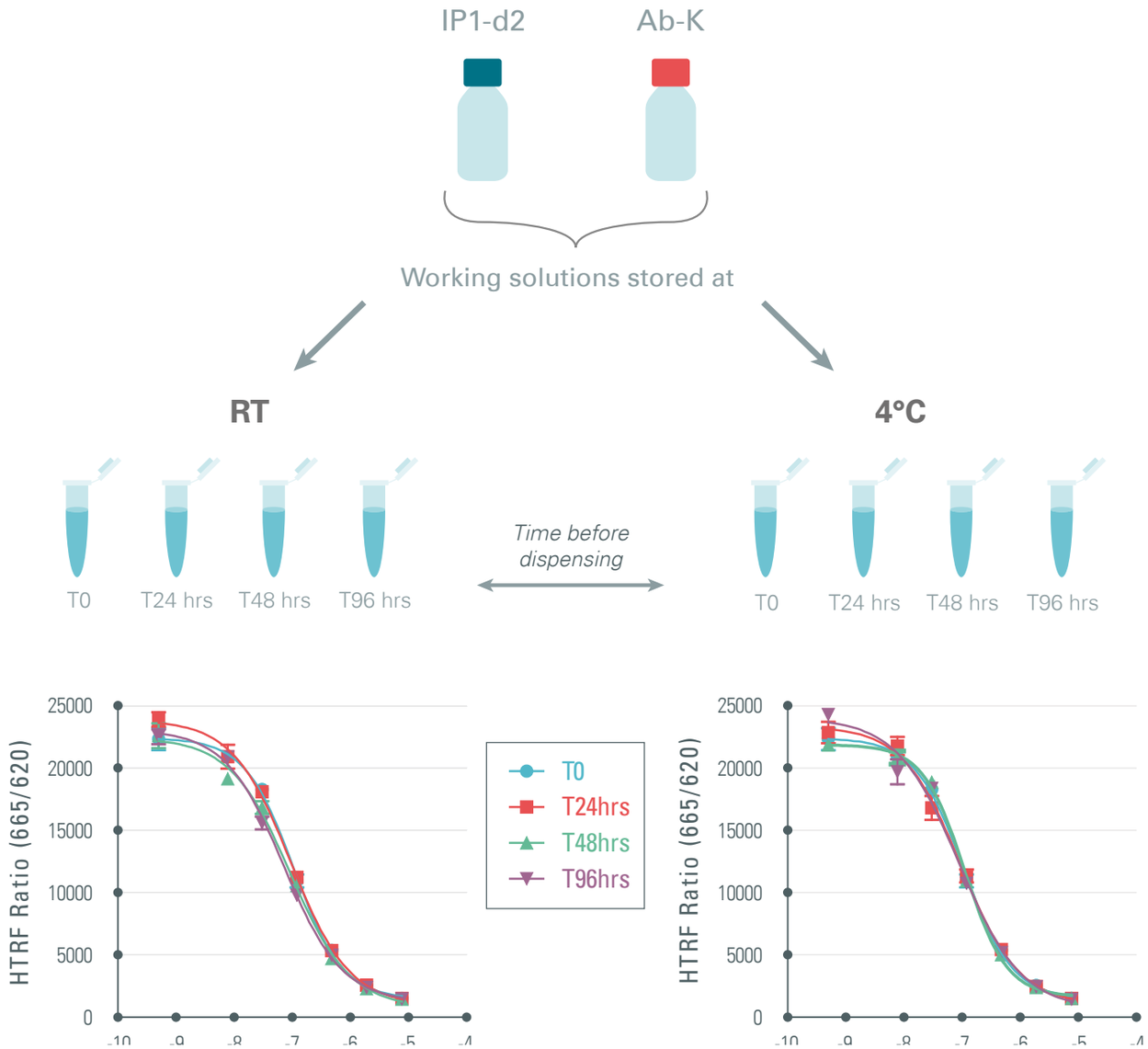

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HIGH THROUGHPUT SCREENING

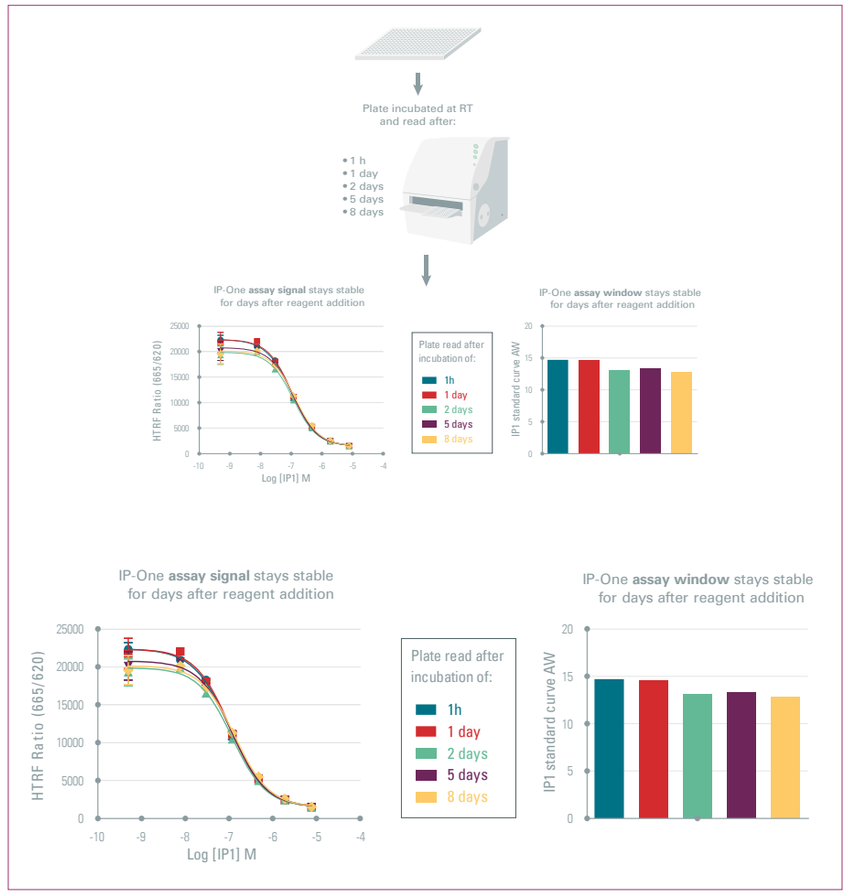
Performance

Stability: Signal stability is key for a successful HTS campaign

An unstable signal means that plates must be read shortly after adding reagent, potentially decreasing throughput. Additionally, in the event of a problem on the screening chain, the signal may be lost forever.

Plates were incubated at room temperature for various lengths of time (from 1 hr. to 8 days) before being read on an HTRF compatible reader. The signal, as well as the IP1 standard curve assay window, remain stable over time, demonstrating the same performance.

- With its stable signal, IP-One protects against unforeseen issues that may occur on the screening platform
- IP-One is highly flexible and allows you to read your results at any point in time



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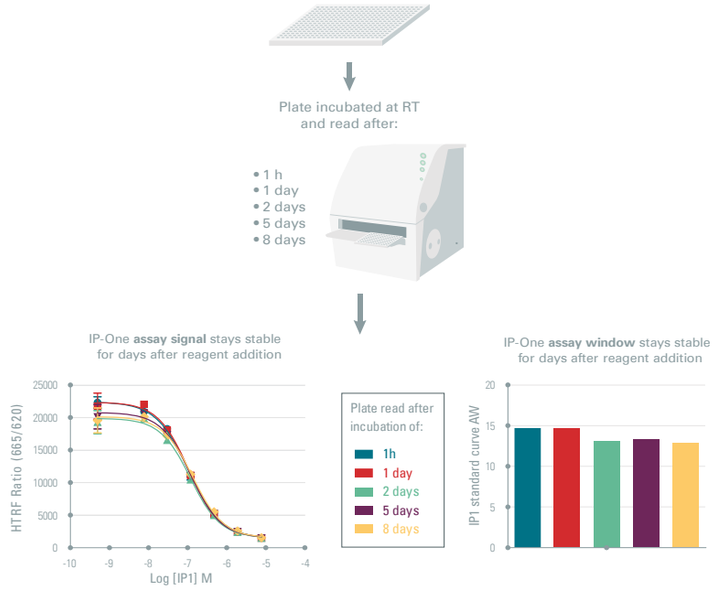

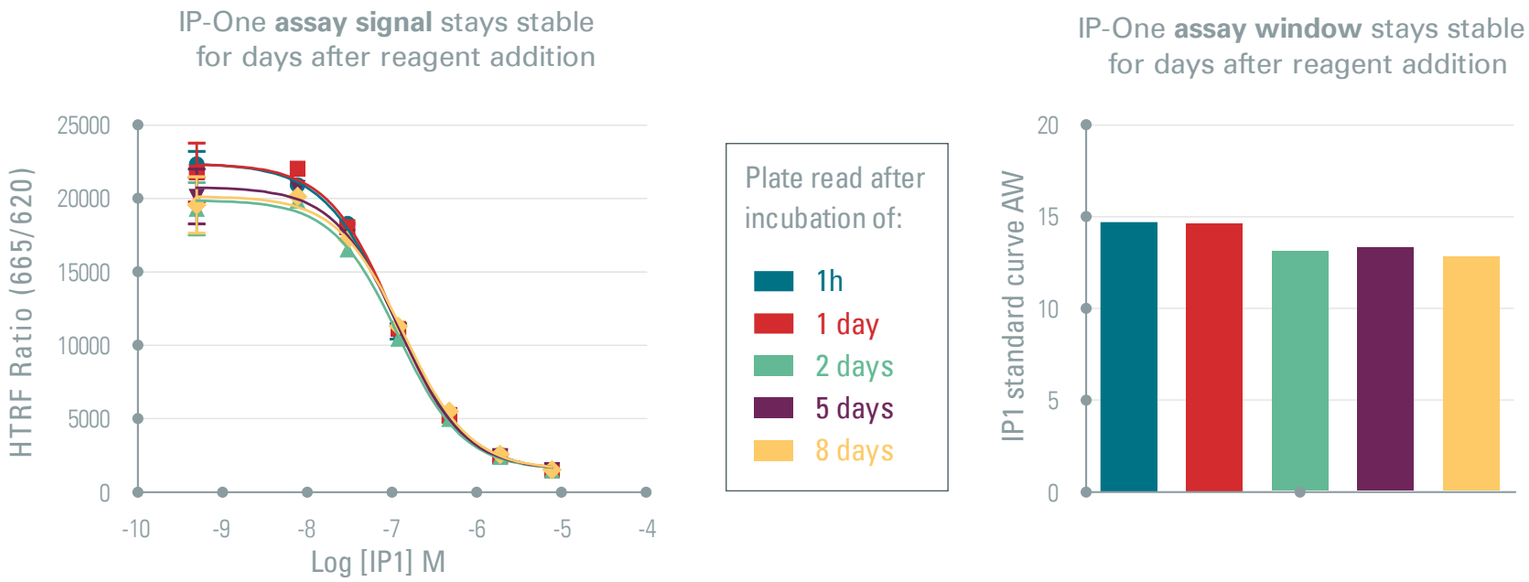

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HIGH THROUGHPUT SCREENING

Reagent Availability

Our kits are always in stock

All IP-One reagents, including the anti-inositol-1 antibody, are produced by Revvity laboratories directly, which means that we can guarantee reagent accessibility and stock availability.

Sourcing multiple batches of reagents affects HTS performance and reproducibility. Revvity understands that having access to a single batch for HTS is a requirement.

Revvity delivers the exact volume of reagent, in a single batch, needed to match your screening requirements.

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HIGH THROUGHPUT SCREENING

Reagent Quality

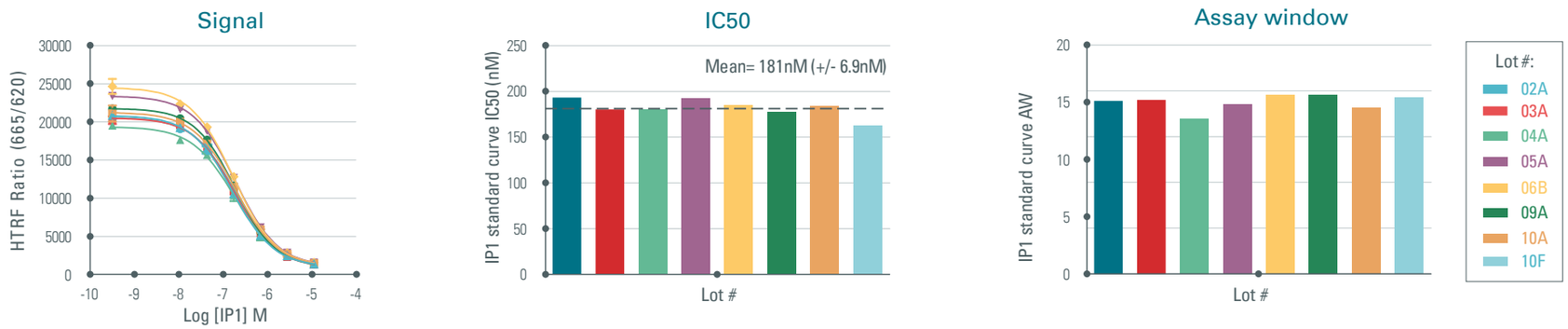
Batch-to-batch quality control

We invest significant resources to guarantee the quality of our screening reagents. All Revvity reagents are required to pass **strict quality metrics**, with every batch tracked for:

- Labeling efficiency
- Assay signal
- IC50 accuracy
- Batch-to-batch reproducibility

We garantee our **quality**

IP1 standard curves (Batch to batch reproducibility):



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THERAPEUTIC AREA

Biased Agonism

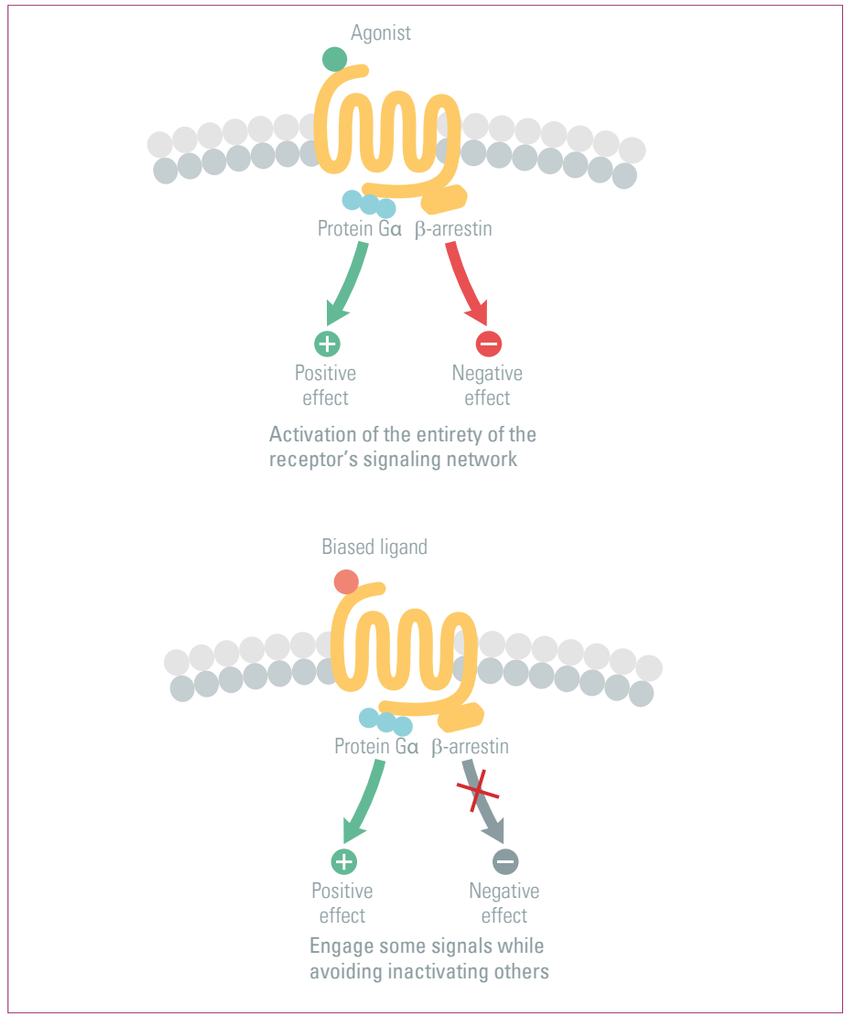
Needs differ with the target field. At Revvity, we know the importance of making assays that adapt to your needs, not the opposite.

IP-One has numerous advantages that will help you in your daily research.

Introduction

GPCR regulation has proven to be far more complex than previously thought by the scientific community. Evidence that GPCRs can couple to several effector pathways, and the existence of biased agonists able to activate them differentially, have introduced a new level of opportunity in GPCR drug research.

"Classical" agonists and antagonists respectively turn on, or off, the entirety of a receptor's signaling network.



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THERAPEUTIC AREA

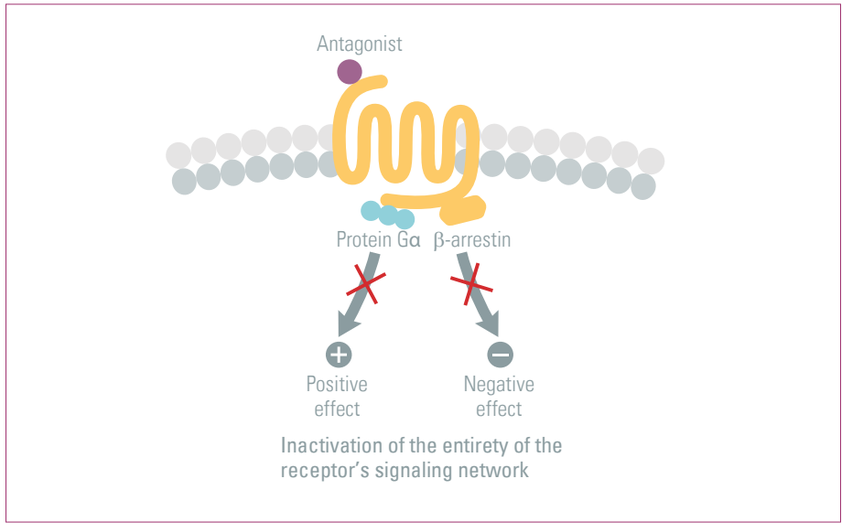
Biased Agonism

Biased ligands introduce a **new way to modulate GPCR function** in a finer manner and to separate therapeutic aspects from side-effects.

Nonetheless, it is still a challenge to identify and qualify ligands that activate only the pathways associated with the requested therapeutic benefit, and reliable tools are required. One interesting method for dissecting GPCR signal transduction concerns the Gαq signaling pathway.

IP-One offers significant advantages over traditional calcium assays including:

- IP-One is a «true equilibrium» assay
- IP-One is specific to Gαq signaling
- IP-One simplifies bias calculation
- IP-One effectively measures a receptor-proximate signal
- IP-One a reliable tool used by leading scientists worldwide



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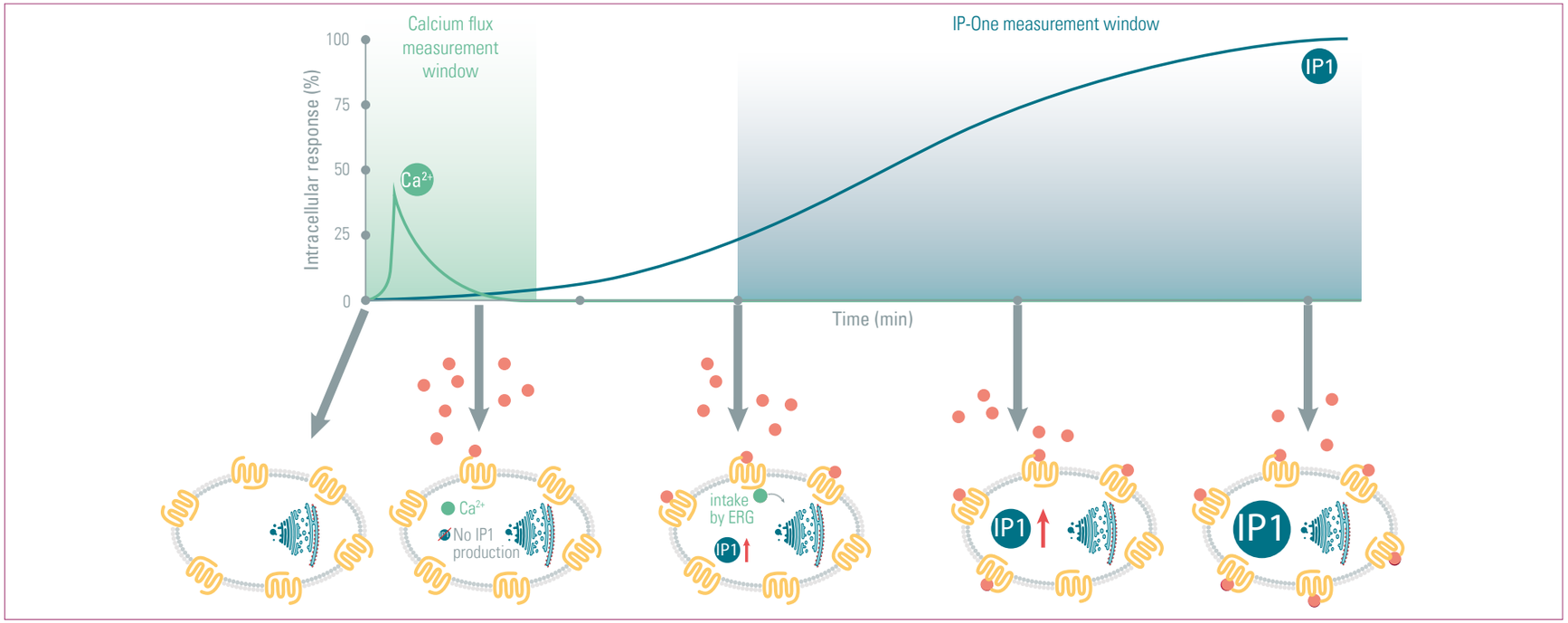
Biased Agonism

IP-ONE is a “true equilibrium” assay

Calcium transient assays are not ideal for characterizing drug bias on Gq coupled receptors. Due to the rapid nature of the calcium signal, flux assays are unlikely to reflect a true equilibrium state, a concept known as hemi-equilibrium. The interpretation of results may be hindered because of the non-equilibrium state. What may appear as functional selectivity could in reality stem from a lack of pre-equilibration. Bias calculation may thus be erroneous in the end.

Built on an accumulation assay, IP-One **distinguishes slow-acting agonists from true-biased agonists easily,** delivering more accurate data.



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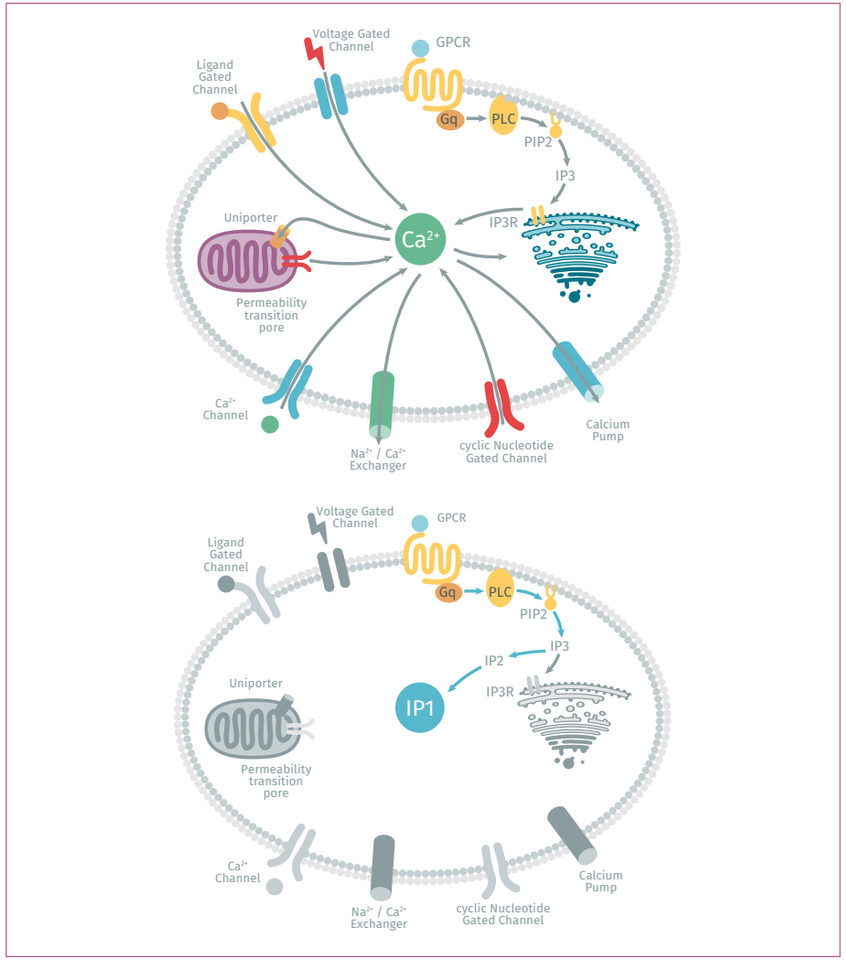
Biased Agonism

IP-ONE is specific to Gαq signaling

Calcium responses are complex in nature. Calcium release is frequently triggered by non-G protein mechanisms, including Ca2+ permeable channels, calcium pumps, and calcium transporters.

IP-One ensures signal specificity. **IP-One only measures IP1,** an analyte that is solely produced by the action of the phospholipase enzyme (PLC) and mediated by GPCRs.

IP-One delivers with reliability and predictability, for results you can trust.



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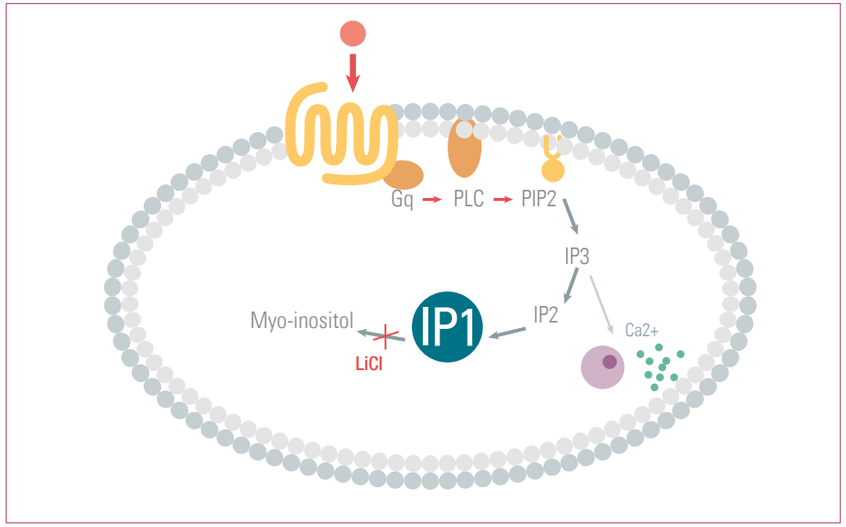
Biased Agonism

IP-One simplifies bias estimation and calculation

Signaling bias is a spatial concept. Bias may occur at different levels of the transduction cascade.

IP1 production occurs at a more proximal level of GPCR signal transduction than calcium release. **Bias estimation can benefit from the measurement of an event close to β-arrestin recruitment in the signal cascade.**

Moreover, calcium assays deliver a fluorescence readout that cannot be converted easily in calcic concentration. The Revvity IP-One assay rapidly delivers compound potency and efficacy in units of IP1 concentrations. The result is a simpler and more straightforward calculation of bias.



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Biased Agonism

Literature

IP-One is a **reliable tool** commonly used by leading scientists and investigators. A significant body of literature has been dedicated to IP-One.

Discover why leading academic authorities have relied on Revvity's IP-One to advance their biased signaling research [1][2][3].

Also read why private companies use IP-One to fuel their own drug development programs [4].

- [1] ***Zimmerman, Brandon, et al***. «Differential β-arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs.», Science Signaling 5.221 (2012): ra33-ra33.
- [2] ***Kenakin, Terry, et al***. «A simple method for quantifying functional selectivity and agonist bias.», ACS Chemical Neuroscience 3.3 (2012): 193-203.
- [3] ***Rajagopal, Sudarshan, et al.*** «Quantifying ligand bias at seven-transmembrane receptors.», Molecular Pharmacology 80.3 (2011): 367-377.
- [4] ***Kim, Ki-Seok, et al.*** «β-arrestin-biased AT1R stimulation promotes cell survival during acute cardiac injury.», American Journal of Physiology-Heart and Circulatory Physiology 303.8 (2012): H1001-H1010.

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PHARMACOLOGY

Compound Characterization

The success of your structure-activity relationships (SAR) campaigns is intimately dependent on the availability of pharmacologically accurate bioassays.

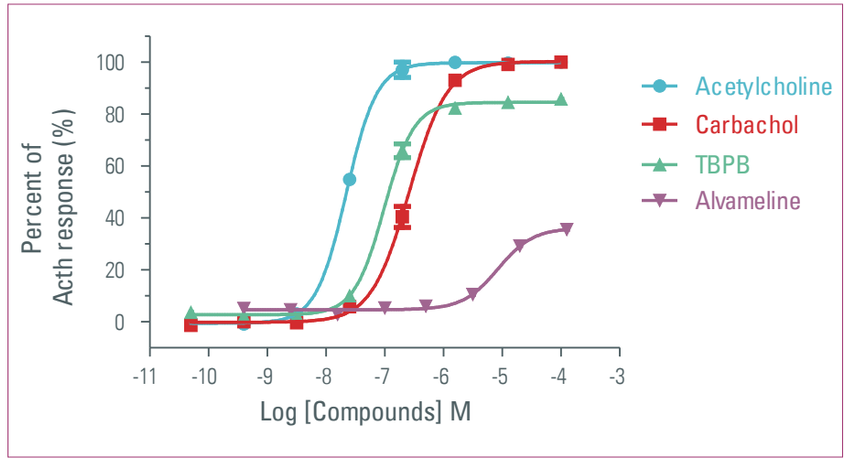
When these assays can reliably and precisely describe the drug receptor interactions involved, the chemical diversity of the leads can effectively be narrowed down and the best drug candidates selected with certainty.

Learn how IP-One delivers unbiased and accurate pharmacological profiles for results you can trust.

Full and partial agonist

This graph shows a typical agonist mode screening assay using a CHO-M1 stable cell line model. Four well-known agonists, Acetylcholine, Carbachol, TBPB, and Alvameline, have been characterized and ranked in terms of potency and efficiency.

The IP-One assay enables perfect characterizations and discrimination of full from partial agonist compounds.



Compounds	Class	Potency (nM)	Efficiency (%)
Acetylcholine	Full agonist	22	100
Carbachol	Full agonist	260	100
TBPB	Partial agonist	100	85
Alvameline	Partial agonist	8500	35

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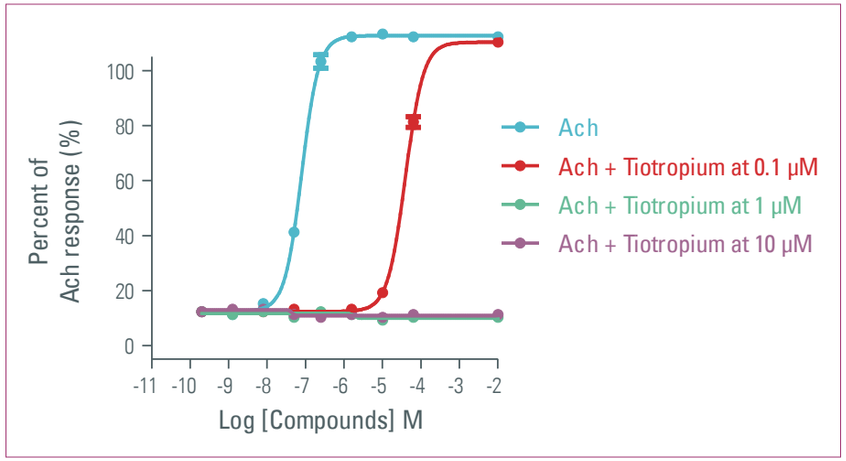
Compound Characterization

Antagonist

Tiotropium is a well-known insurmountable antagonist which binds on the orthosteric site of the M1 receptor.

Using a CHO-M1 stable cell line, the acetylcholine full agonist is incubated with or without different doses of tiotropium (0.1µM, 1µM, 10µM).

The results show a typical insurmountable antagonist pattern using the IP-One assay, as described in the literature*.



Compounds	EC50 (nM)
Acetylcholine	79
Acetylcholine + Tiotropium at 0.1 µM	3971
Acetylcholine + Tiotropium at 1 µM	Complete inhibition
Acetylcholine + Tiotropium at 10 µM	Complete inhibition

** Peter J. Barnes, MA, DM, DSc.* The Pharmacological Properties of Tiotropium

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PHARMACOLOGY

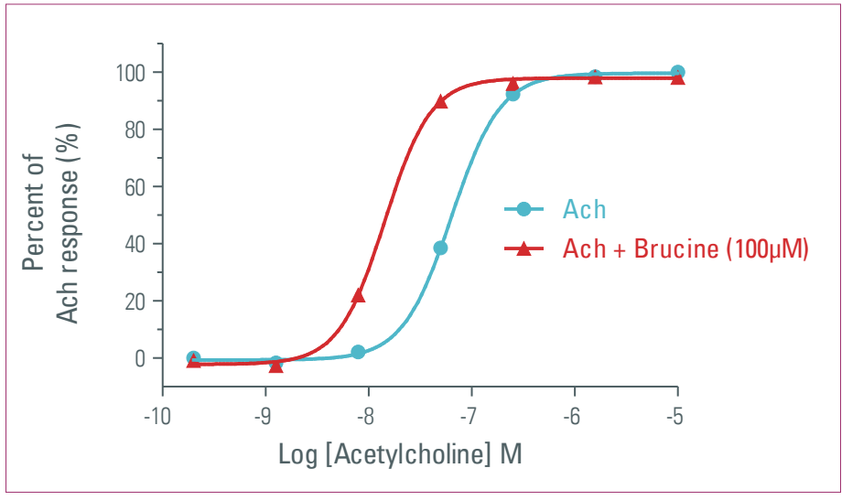
Compound Characterization

Allosteric modulators

Brucine, a well-known PAM of the Muscarinic M1 receptor, was well characterized using the IP-One assay.

The CHO-M1 stable cells were treated with the acetylcholine full agonist alone or in addition to the Brucine positive allosteric modulator. Brucine acts by increasing the potency of the acetylcholine to the M1 receptor 4.6 fold at 100µM. and efficacy in units of IP1 concentrations.

The result is a simpler and more straightforward calculation of bias.



Compounds	Class	Potency of Ach (nM)	PAM effect
Acetylcholine	Full agonist	65	-
Acetylcholine + Brucine (100 µM)	Full agonist + PAM	14	4.6 fold

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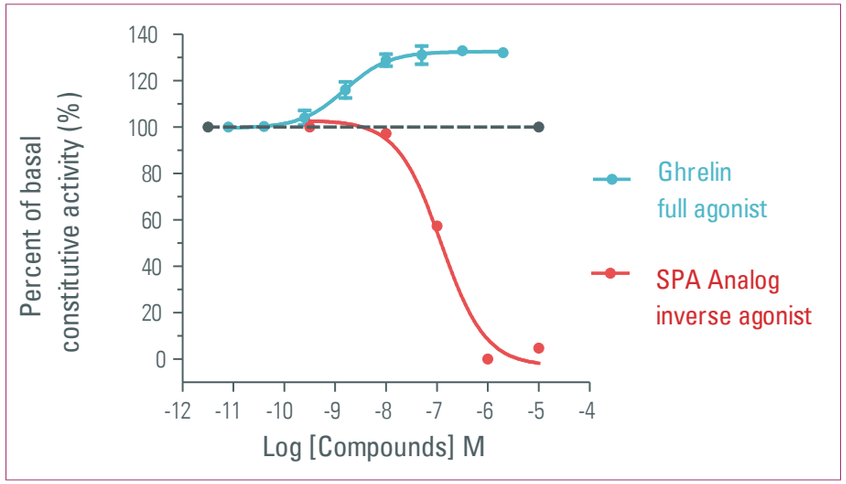
Compound Characterization

Inverse agonist

The graph shows the profiling of a well-known inverse agonist of the GHSR1a receptor: SPA analog.

This compound inhibits the constitutive activity of the ghrelin receptor with high efficiency, reaching a level of no remaining constitutive activity (0%) after treatment.



Compounds	Class	Potency (nM)	% of basal constitutive activity
Acetylcholine	Full agonist	22	132
SPA analog	Inverse agonist	260	0

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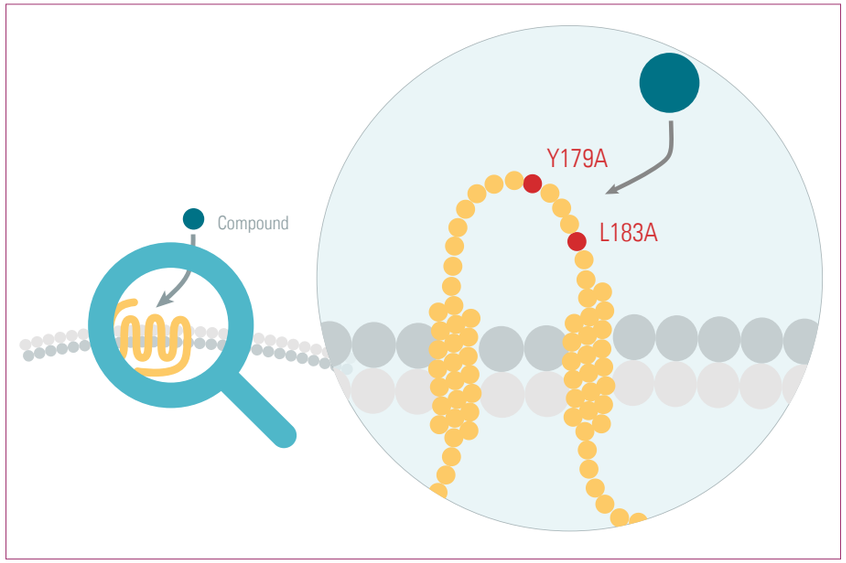
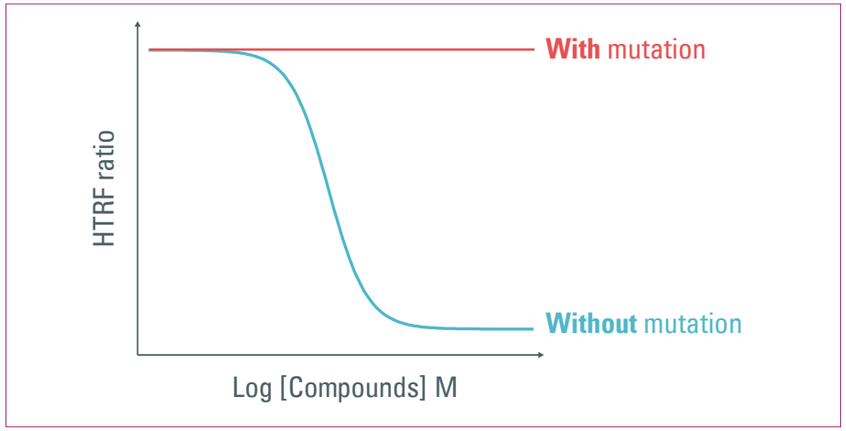
Alanine Screens

Studying compound pharmacology on alanine mutants

IP-One is the preferred tool to study compound pharmacology on alanine mutants and will help you:

- Identify receptor selective ligand and understand the molecular basis of selectivity[1]
- Identify orthosteric and allosteric ligand binding pockets
- Understand receptor determinants of potency and efficacy
- Obtain functional validation of computational modeling of ligand binding





[1] ***Abdul-Ridha, Alaa, et al.*** «Molecular determinants of allosteric modulation at the M1 muscarinic acetylcholine receptor.», Journal of Biological Chemistry 289.9 (2014): 6067-6079.

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- *Rovira, Xavier, et al.* «Overlapping binding sites drive allosteric agonism and positive cooperativity in type 4 metabotropic glutamate receptors.», The FASEB Journal 29.1 (2015): 116-130.
- *Siddiquee, K., et al.* «The apelin receptor inhibits the angiotensin II type 1 receptor via allosteric trans-inhibition.», British journal of pharmacology 168.5 (2013): 1104-1117.
- *Kuohung, Wendy, et al.* «A high-throughput small-molecule ligand screen targeted to agonists and antagonists of the G-protein-coupled receptor GPR54.», Journal of biomolecular screening 15.5 (2010): 508-517.
- *Matsufuji, Tetsuyoshi, et al.* «Discovery of novel chiral diazepines as bombesin receptor subtype-3 (BRS-3) agonists with low brain penetration.», Bioorganic & medicinal chemistry letters 24.3 (2014): 750-755.
- *Nordqvist, Anneli, et al.* «New hits as antagonists of GPR103 identified by HTS.», ACS medicinal chemistry letters 5.5 (2014): 527-532.
- *Yoshikawa, Keita, et al.* «Identification of alpha-substituted acylamines as novel, potent, and orally active mGluR5 negative allosteric modulators.», Bioorganic & medicinal chemistry letters 25.16 (2015): 3135-3141.

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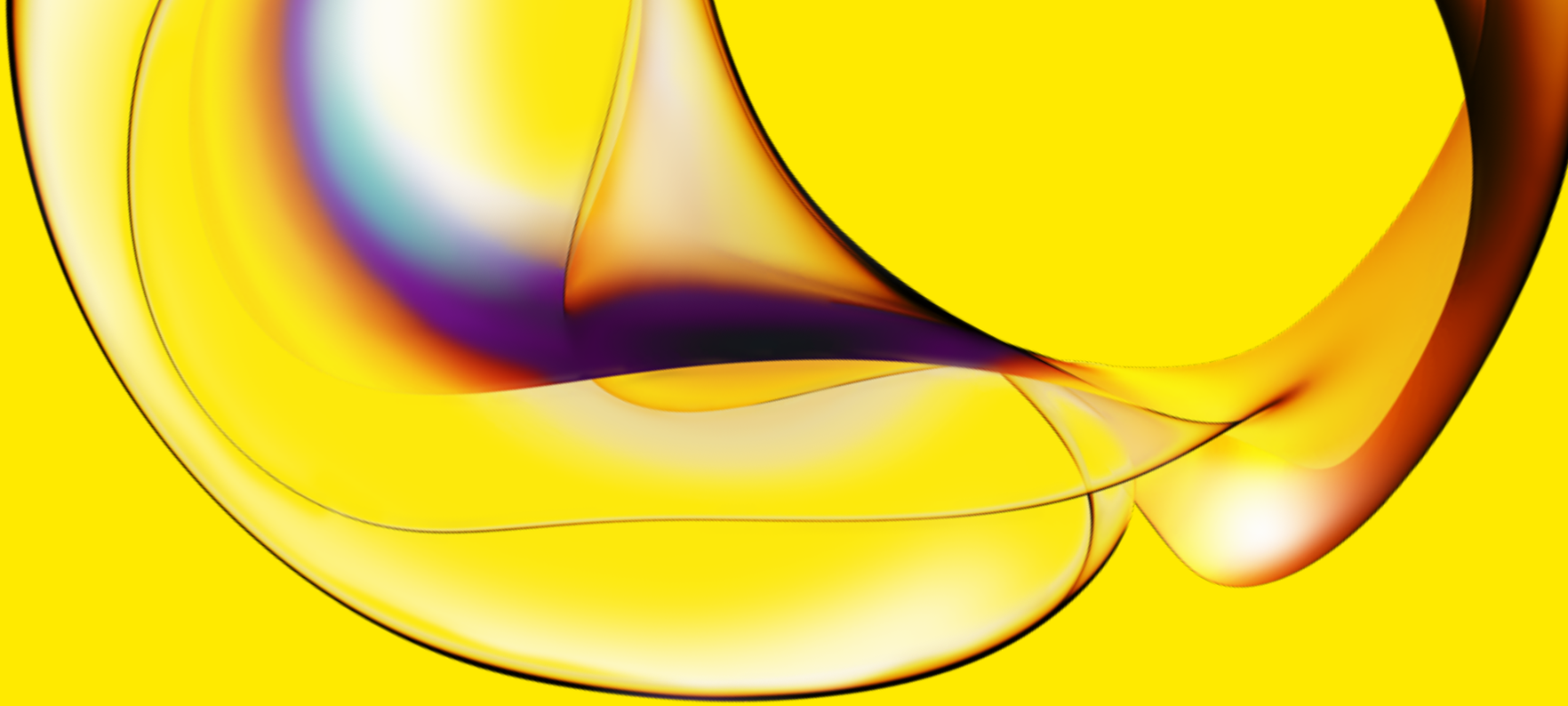

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