

# Uncovering HTS-grade cellular assays for protein-protein interactions

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## PPI as target for drug discovery

**Protein-protein interactions (PPIs) are key processes** in numerous biological functions and detecting PPI events is not only essential for understanding physio- and pathophysiological pathways but also fundamental for **developing new therapeutics**.

Here we describe a **case study in which PPI**, between a transmembrane protein and its cytoplasmic partner, **were successfully investigated** both in intact and viable cells and in a biochemical, cell-free format using cell lysates.

Applying an optimized Lytic Protocol, a successful **screening campaign was conducted** on a homogeneous preparation of proteins extracts, knowing that this tactic would have been suitable for both impermeable and cell membrane permeable compounds.

**Axxam**, by mean of its consolidated experience and expertise, contributes to the **development of functional cell-based assays for many different target classes**, applying multiple and diversified technologies, including **fluorescent dyes- and substrates-, genetically encoded sensors-, optogenetic-, electrophysiology-, radiometric- and imaging- based detection methods**.

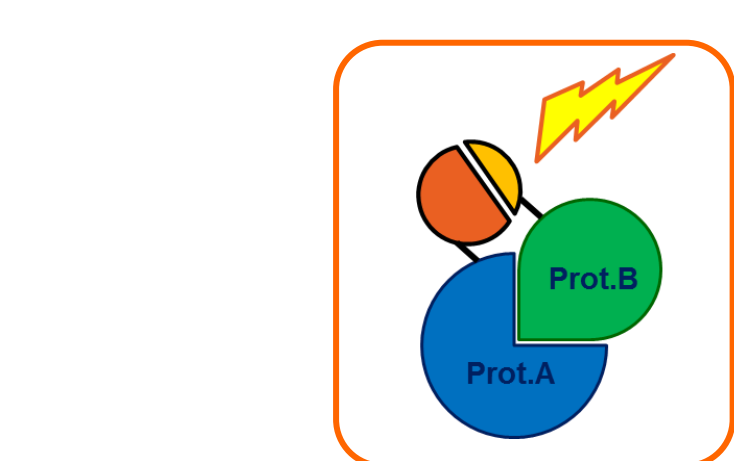
The **development of functional cell-based assays, suitable for high-throughput screening campaigns**, represents a fundamental approach to **discover novel inhibitors and activators of diseases-related targets**, for therapeutic purposes.

## NanoBiT Protein:Protein Interaction System

To develop a **PPI-assay**, suitable for HTS activities, we took advantage of the complementation reporter assay called **NanoLuc Binary Technology (NanoBiT)**.

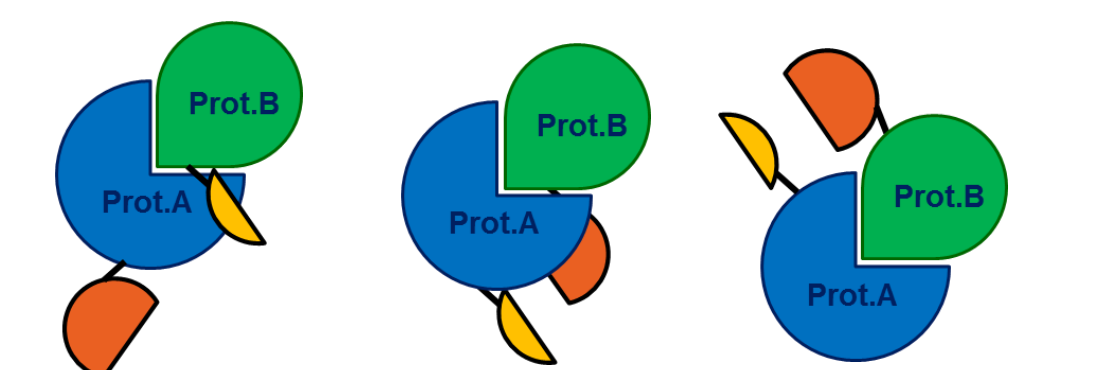
The luciferase **NanoLuc** is divided in two subunits, "Large BiT" and "Small BiT", which interact very weakly; when **fused to proteins of interest**, they reconstitute an **active and stable luciferase only upon the interaction of the latter**.

The NanoLuc is split into a Large (LgBiT) and a Small subunit (SmBiT)



PPI brings LgBiT and SmBiT together, reconstituting a functional NanoLuc

LgBiT and SmBiT are conjugated with the two interactors, evaluating all possible combinations

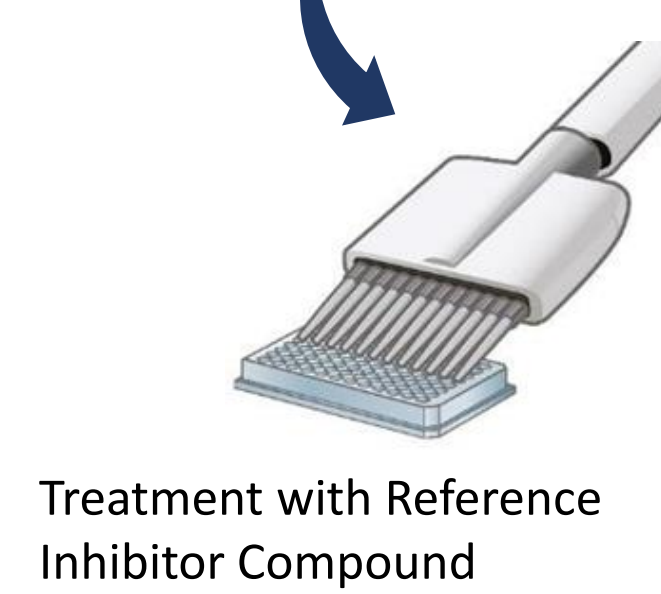
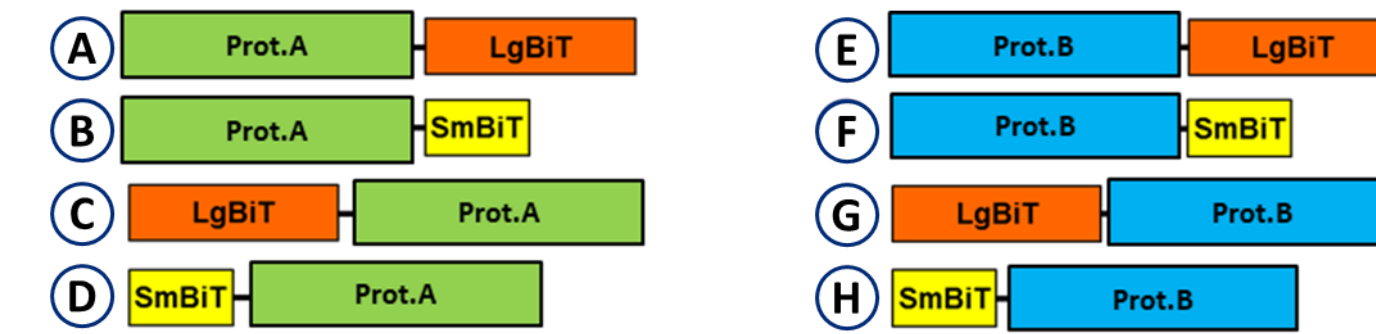


Not all LgBiT and SmBiT fusions are expected to work

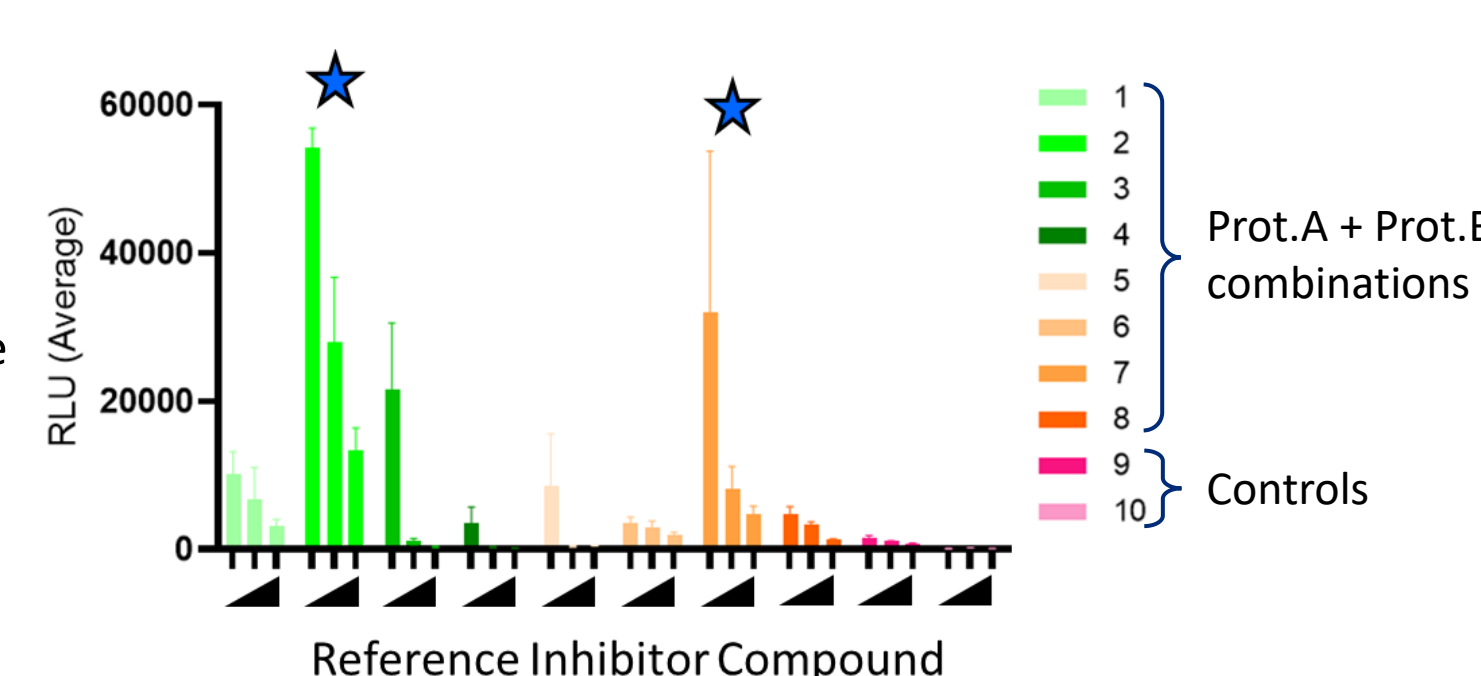
## Proof-of-Principle activities

The NanoBiT technology was successfully applied for the study of PPI between the two targets of interest. In the first instance, we performed transient transfections of **all the different combinations** of N- or C-terminal LgBiT and SmBiT fusion proteins and **identified the best pairing constructs for PPI assays**.

Cells transient transfection with all the possible vectors combinations



Cells lysis and incubation with NanoBiT substrate

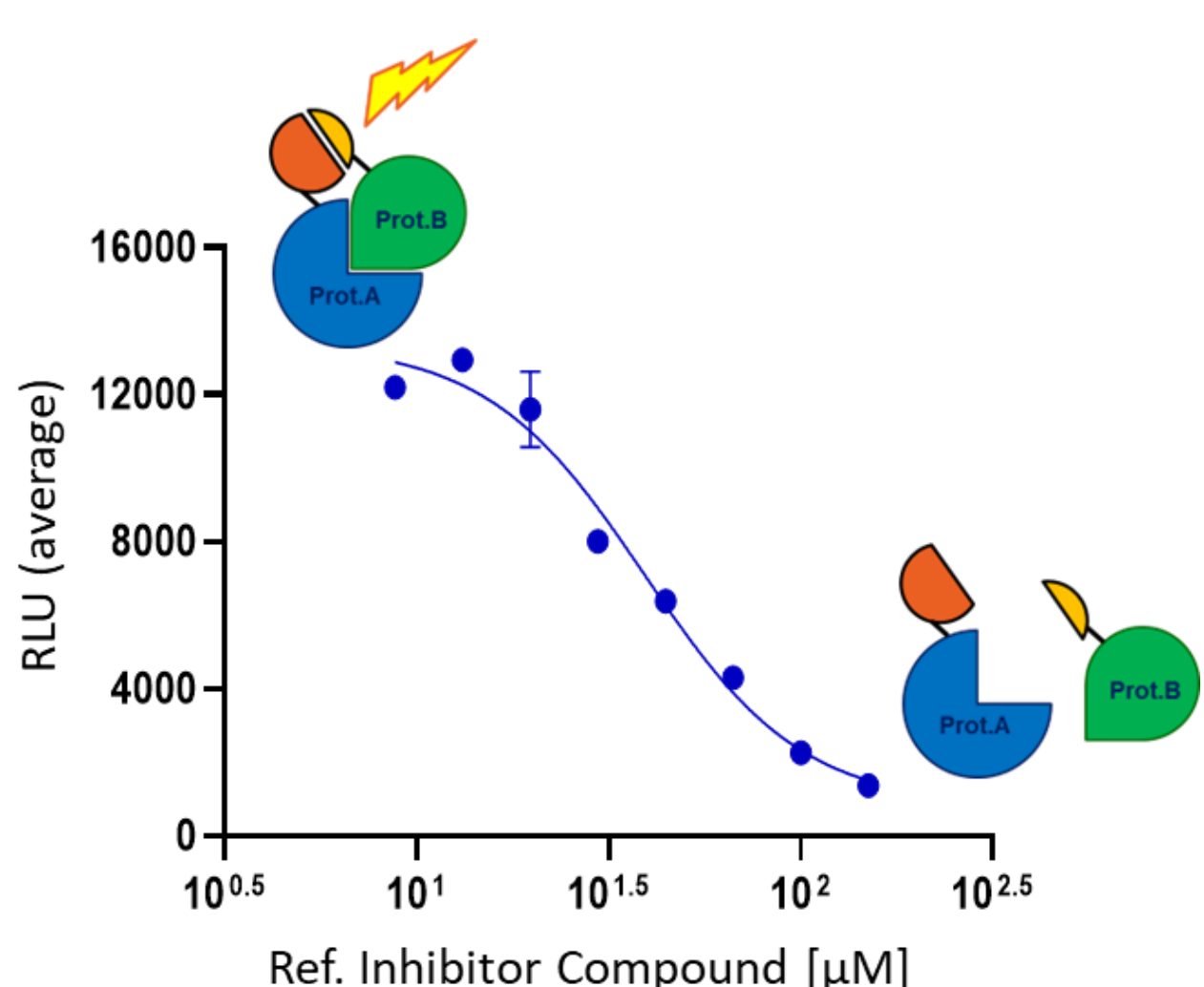
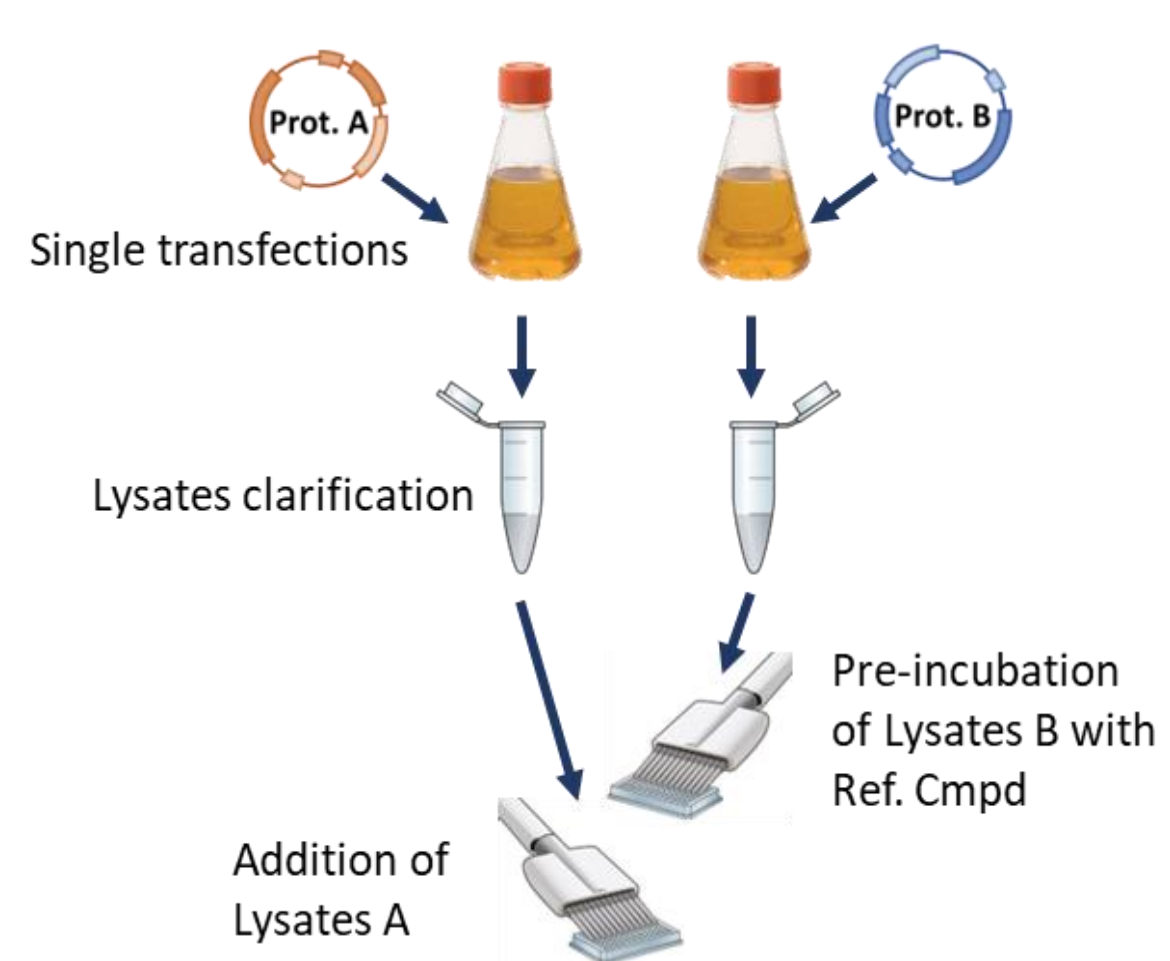


From there, we carefully **optimized and validated two distinct approaches** characterized by the production of cell extracts or by the analysis of viable and intact cells, **Lytic and Live Cell Protocol**, respectively.

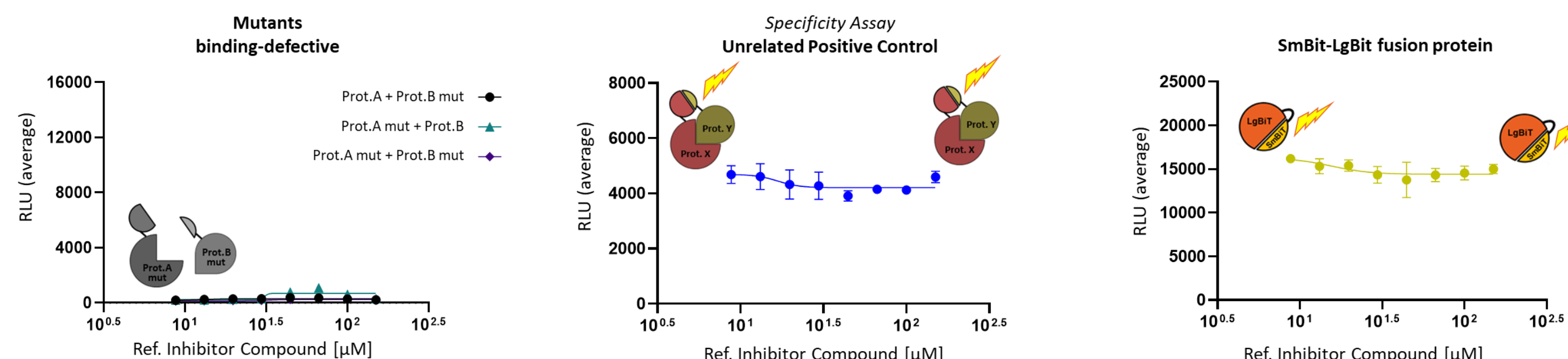
## Lytic Protocol development and optimization

An **accurate assay development and optimization** was performed in order to **obtain a robust and reproducible protocol**, which involves harvesting of transfected Expi293F cells, lysis by sonication, incubation with compounds and addition of the NanoBiT Substrate for detection of interaction.

The obtained assay **satisfied all the Axxam quality criteria for screening purpose**, such as miniaturization, specific response, significant signal-to-background window, RZ' factor  $\geq 0.5$ , signal stability and pharmacology reproducibility in independent experiments.



Several **different controls**, positive and negative, were developed and tested in parallel to the Primary Assay, to confirm **specificity and reliability** of the detected luminescence signal.



Known binding-defective mutants of both Prot.A and Prot.B were expressed in different combination. No signal was detectable, confirming specificity of the measured signal.

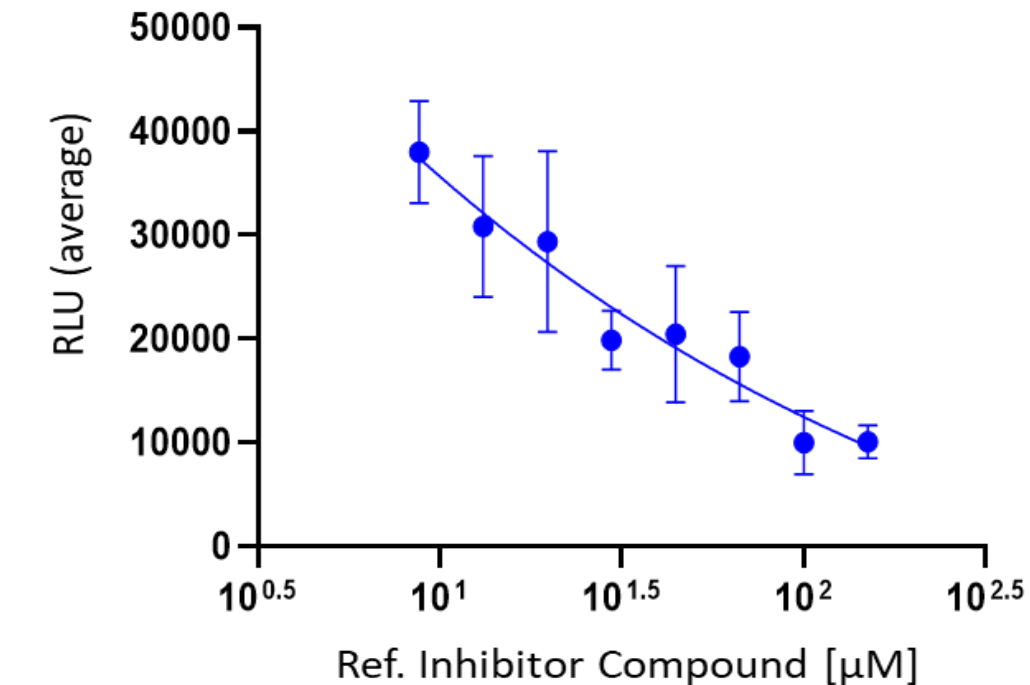
Two interacting proteins, unrelated from the targets, were tagged with LgBiT and SmBiT, validated and used as Specificity Assay during screening, to confirm specificity of Ref. Inhibitor Compound.

A SmBiT-LgBiT fusion protein was also transfected. No effects were detectable on this control of the read-out, upon incubation with the Ref. Inhibitor Compound.

## Live Cell Protocol development

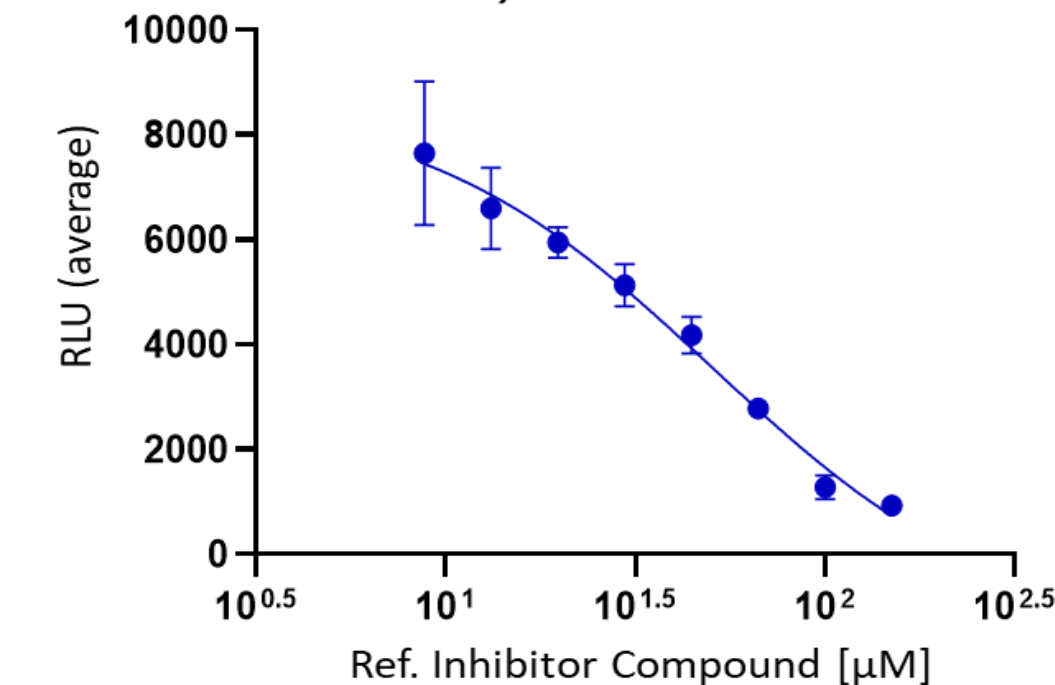
The **best pairing constructs for PPI assay** was used to **develop and validate a Live Cell Protocol**. This cell-based assay allows the identification of small molecules that mediate PPI inhibition, and the analysis of the targets in their physiological environment. Two different approaches were validated, briefly:

Whole Cells Live Protocol



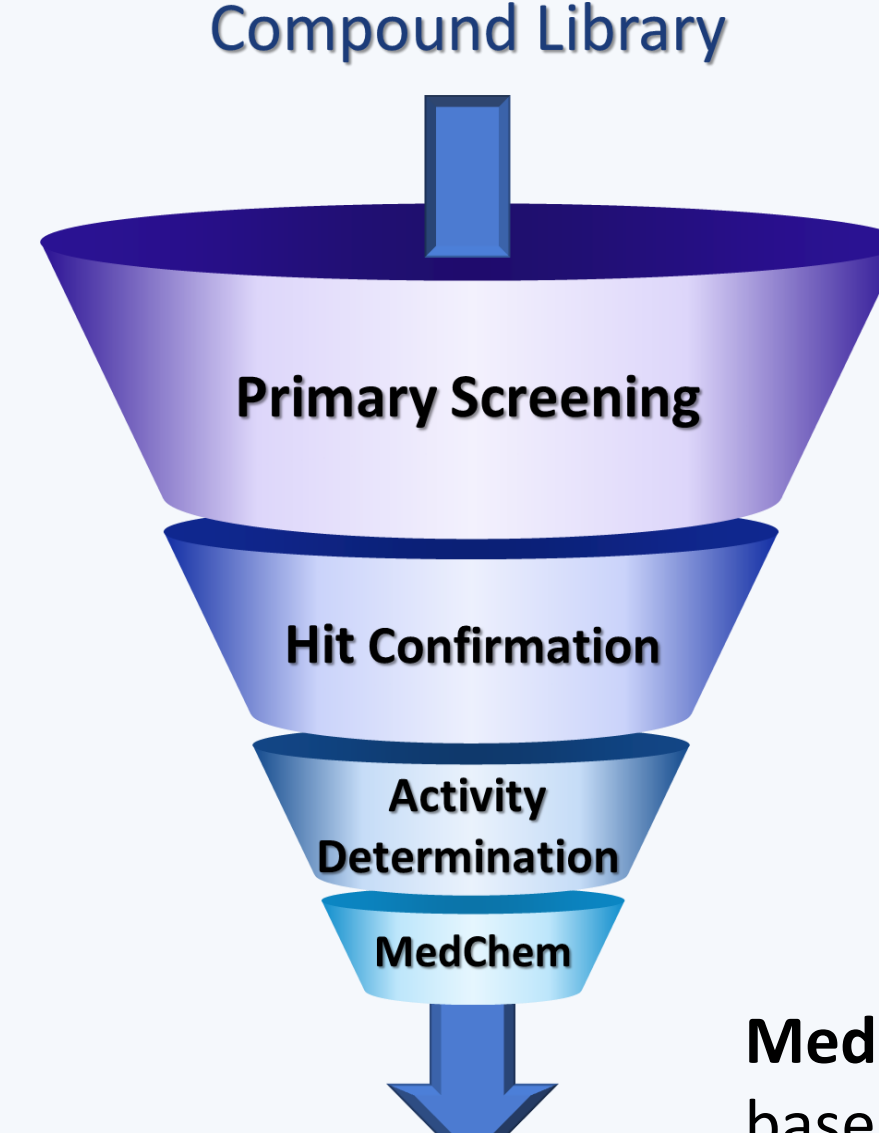
Cells were transfected with tagged Prot.A and Prot.B and seeded in 384w plate. The day after, cells were incubated with compound. At the end of treatment, the cell-permeable NanoBiT Substrate was added for detection of interaction.

Whole Cells Lytic Protocol



Cells were transfected with tagged Prot.A and Prot.B and seeded in 384w plate. The day after, cells were incubated with compound. At the end of treatment, a lysis buffer was added together with NanoBiT Substrate for detection of interaction.

## Screening Funnel



**Primary Screening** was performed on  $\approx 350,000$  cpds from **AXXDiv4.0 library**, in Primary assay

- Majority of plates ( $> 97\%$ ) pass QC criteria of  $RZ' \geq 0.5$
- Signal-to-background on average 9-fold
- Pharmacology of Reference Compound highly reproducible
- The hit rate for Primary Screening is  $\approx 1\%$

**Hit Confirmation** was performed on **primary hits compounds**

- Cpds were tested at 2 conc., against Primary and Specificity assays
- $\approx 2,500$  hits confirmed their specific activity against Primary assay, corresponding to a good confirmation rate of  $\approx 75\%$

**Medicinal Chemistry review**: hit selection for further characterization based on **patentability, druglikeness and chemical tractability**

## Conclusion

We developed, characterized, and validated two different NanoBiT-based assays, that allowed the identification of small molecules inhibiting intracellular PPI between two target proteins.

Lytic Protocol has been employed to run, and successfully conclude, an HTS campaign with more than 350'000 compounds.

