# CITRON KINASE: THE TASTY JUICE OF THE EARLY DRUG DISCOVERY

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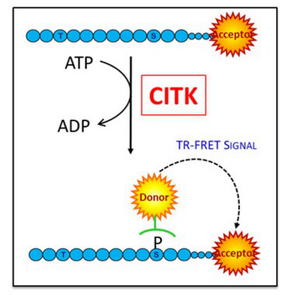
## INTRODUCTION

- **Citron kinase** (**CITK**) is a ubiquitous **serine/threonine kinase** associated to the active form of RhoA small GTPase and involved in actin remodeling and cytokinesis.
- CITK has a key role in cell division and in diseases such as **tumors** and **brain diseases**. For this reason, it represents an attractive target for the identification of small molecules capable of inhibiting its catalytic activity with the aim of developing a successful therapy for the aforementioned pathologies.
- In this work we describe a successful **hit discovery program** performed on CITK kinase activity **using a functional TR-FRET assay** suitable for high throughput screening (HTS) and **screening a focused library of 10,797 compounds** in search of **CITK inhibitors**.

## ASSAY PRINCIPLE



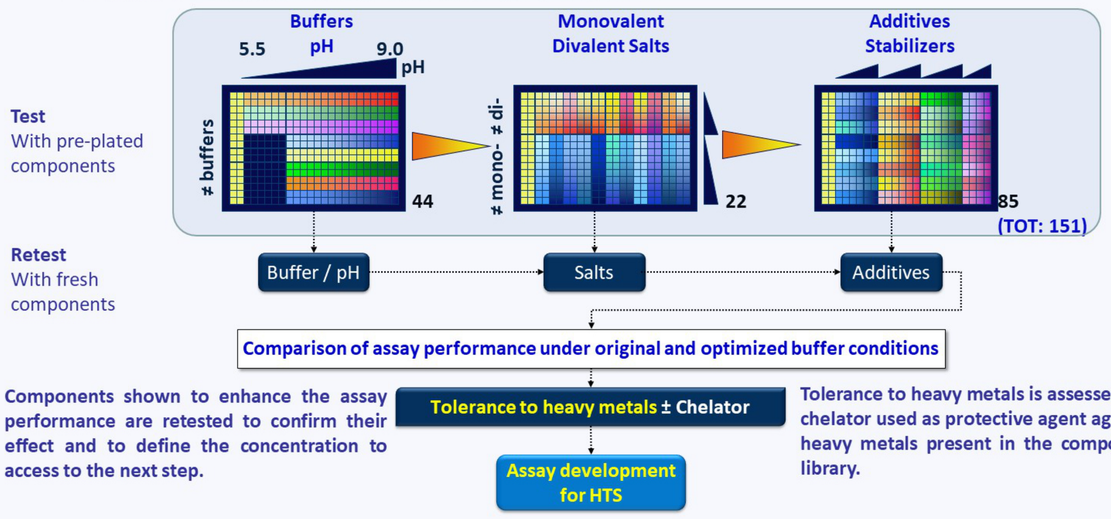

The **TR-FRET assay on CITK kinase activity** is based on the detection of the phosphorylation in serine of a surrogate peptide substrate labeled with an acceptor dye in combination with a specific anti-phospho-serine antibody (***OFF system*** for inhibitors).

## PRIMARY ASSAY DEVELOPMENT AND OPTIMIZATION

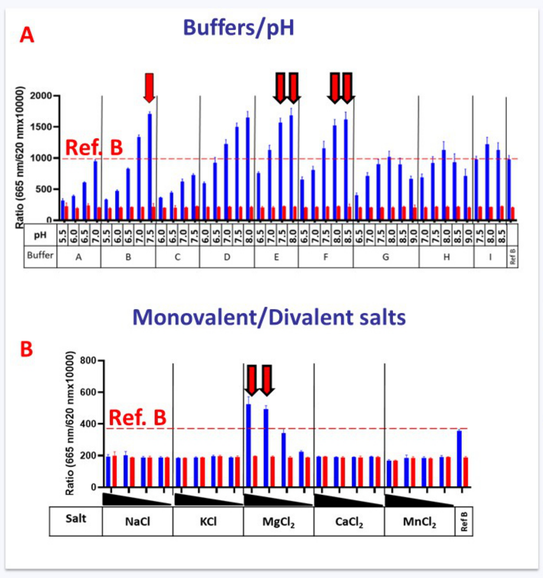
### Buffer optimization workflow

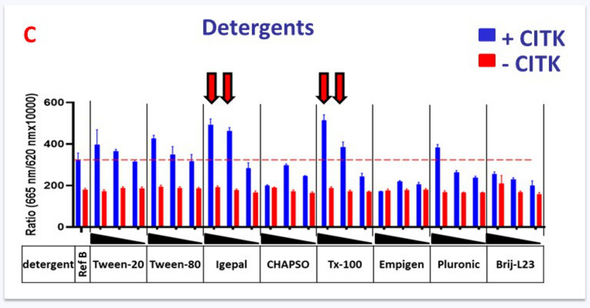
**Goals:**

A. In depth characterisation of the enzymatic activity
B. Improvement of the assay performance and smooth assay adaptation to automation
C. Reduction of the working concentration of the key assay components



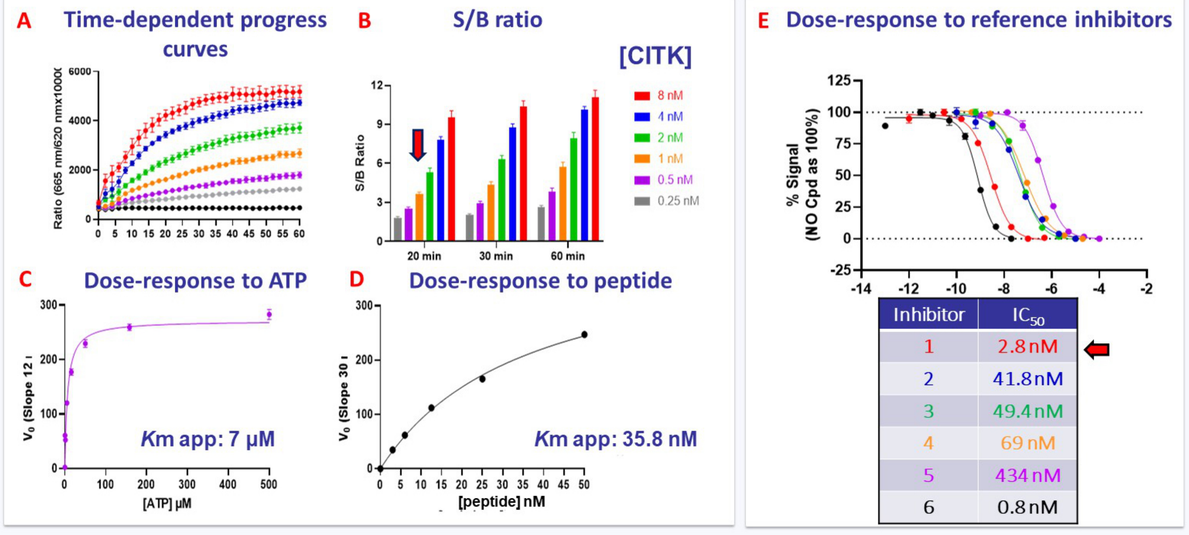

### Optimization of buffer condition




- CITK assay displayed a dependence on **pH 7.5-8.5** (panel A) and on **$MgCl_2$** concentration (panel B).
- **Non-ionic detergents improved TR-FRET signal** (panel C, best results indicated by arrows).
- Under optimized buffer condition the CITK assay showed a **2/3-fold improved performance** (not shown)

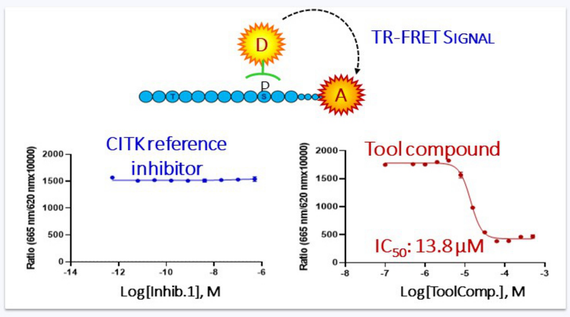
### Development of a primary assay for HTS




| Inhibitor | $IC_{50}$ |
|---|---|
| 1 | 2.8 nM |
| 2 | 41.8 nM |
| 3 | 49.4 nM |
| 4 | 69 nM |
| 5 | 434 nM |
| 6 | 0.8 nM |

- The CITK reaction displayed time-, enzyme-, substrate-, inhibitor-dependency (panel A-E)
- CITK assay for HTS was configured at: **1 nM CITK, ≈ $^{a}K_m$-equivalent substrate concentrations, 20 minutes reaction time** (**S/B** = **3.6**, panel B, see arrow)
- **Inhibitor 1** was selected as reference for HTS (**$IC_{50}$: 2.8 nM**, see arrow in panel E)

## INTERFERENCE ASSAY DEVELOPMENT




- The **interference assay** was insensitive to CITK reference inhibitor (left panel).
- **Tool compound** inhibited the interference assay in a dose-dependent manner (**$IC_{50}$: 13.8 nM**, right panel)

## HIGH-THROUGHPUT SCREENING (HTS)

❑Full automation
❑Pharmacology validation – Reagent stability
❑Multiplate test (5 plates in 2 independent runs)
❑S/B , RZ', CV%
❑Assay performance evaluation

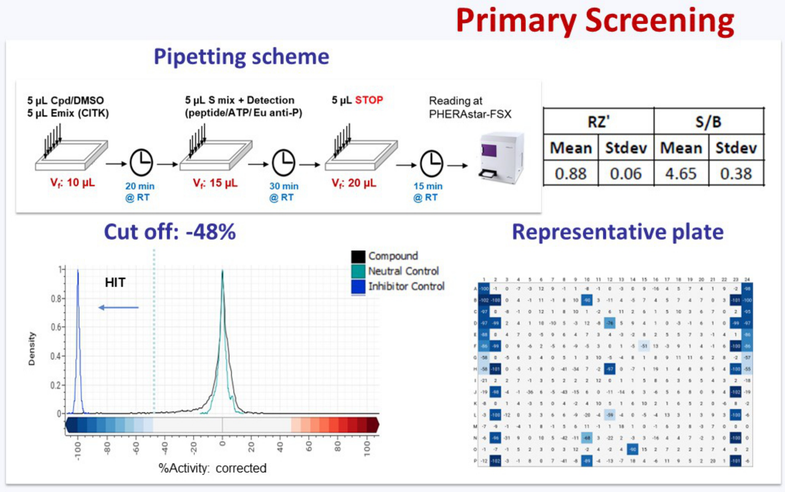
❑**10,797 compounds** from a **focused library**
❑Single concentration (**10 µM**)
❑Single data point (**n=1**)
❑On **primary assay**
❑Data analysis with GeneData Screener® software

**Adaptation to automation of the HTS assays** → **Pilot Screening** → **Primary Screening** → **Potency Determination ($IC_{50}$)**

❑Single concentration (**10 µM**)
❑Triplicate data points (**n=3**)
❑Two separate runs
❑On **960 compounds**
❑On **primary** and **interference assay**

❑**640 compounds**
❑Inter-plate dose response
❑Triplicate data points (**n=3**)
❑**8 concentrations** (from **30 µM**, dil. 1:3,14)
❑On **primary** and **interference assay**

### Primary Screening

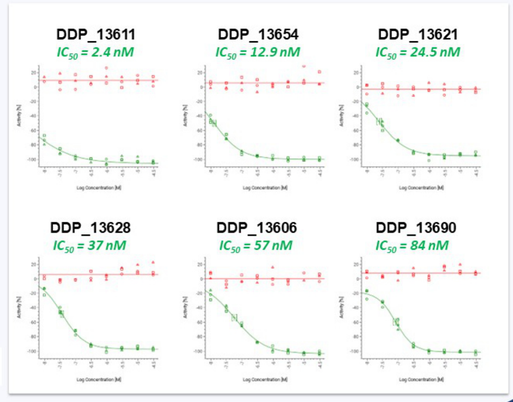



| RZ' | | S/B | |
|---|---|---|---|
| Mean | Stdev | Mean | Stdev |
| 0.88 | 0.06 | 4.65 | 0.38 |

- The **hit rate** was **2.6%** (statistical cut-off: 48% inhibition), for a total of **282 primary hits**.
- Applied quality criteria:
  - **RZ' ≥ 0.5**
  - **Absence of any uncorrectable signal variability** (i.e., edge effect, drift, patterns) across each single plate.

### Potency Determination

- **427 compounds** out of 640 were confirmed as putative **specific CITK inhibitors**.
- **10 compounds** displayed **$IC_{50}$ values <100 nM** (6 shown).




## CONCLUSIONS

- Development and optimization of a **functional TR-FRET-based CITK assay** in **384 MTP format**
- Screening campaign (on primary and interference assays) → **427 specific CITK inhibitors.**
- **Valid early drug discovery strategy** for identification of lead compounds potentially useful for treatment of pathologies such as cancer and brain diseases.

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