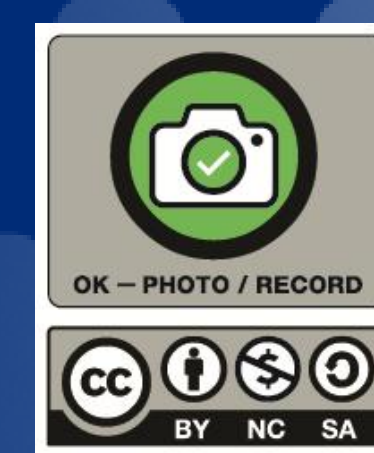


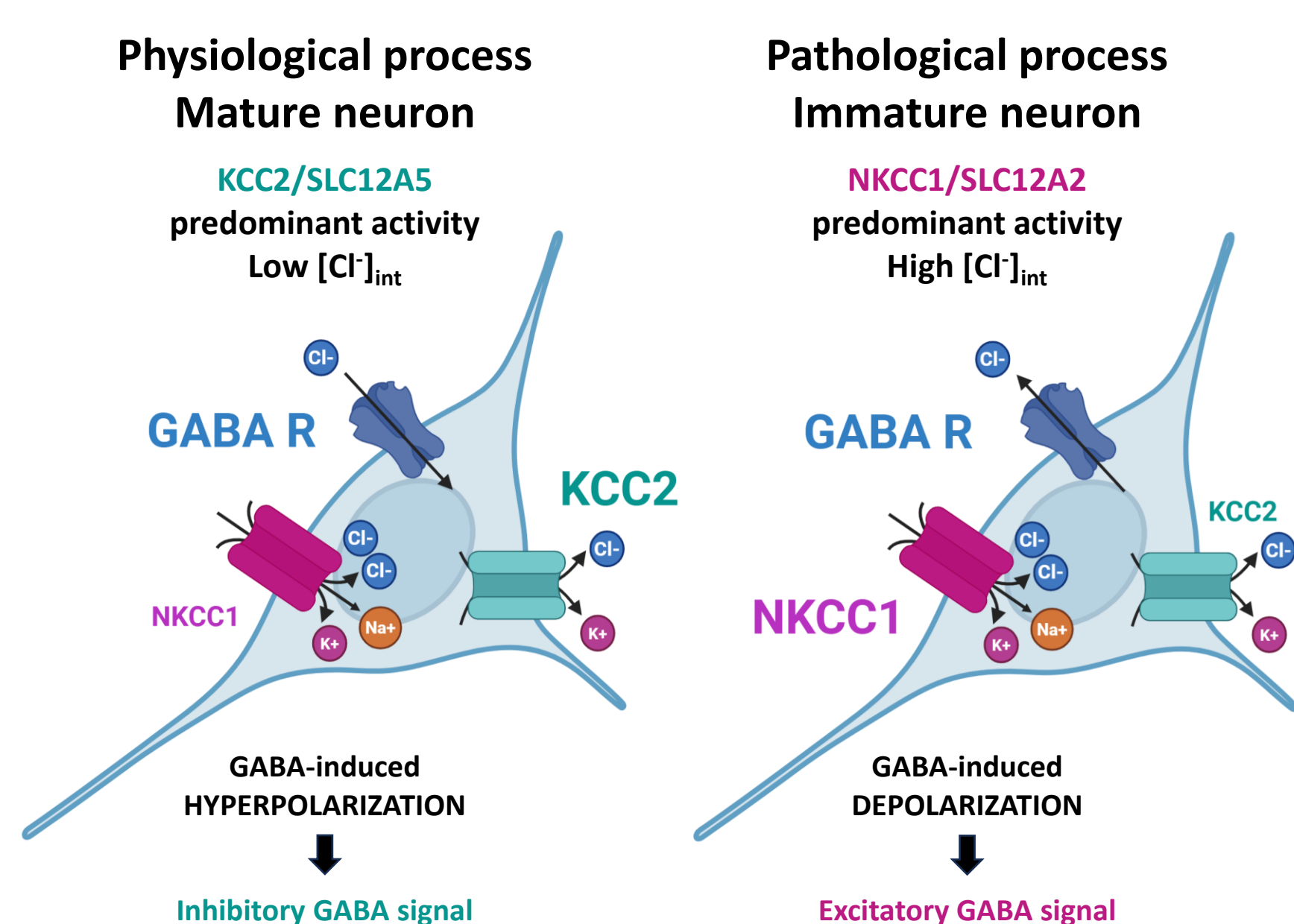
Advancing KCC2 Pharmacological Potentiation: Breaking the State-of-the-Art for CNS Drug Discovery

Federica Naso¹, Giovanna Maresca¹, Tiziana Ada Renzi¹, Manuel Cernigoj¹, Alessandro Poli¹, Samuel Bradberry¹, Loredana Redaelli¹, Jean-François Rolland¹
¹Axxam S.p.A. OpenZone, Via Meucci 3, 20091 Bresso (Milan), Italy



INTRODUCTION

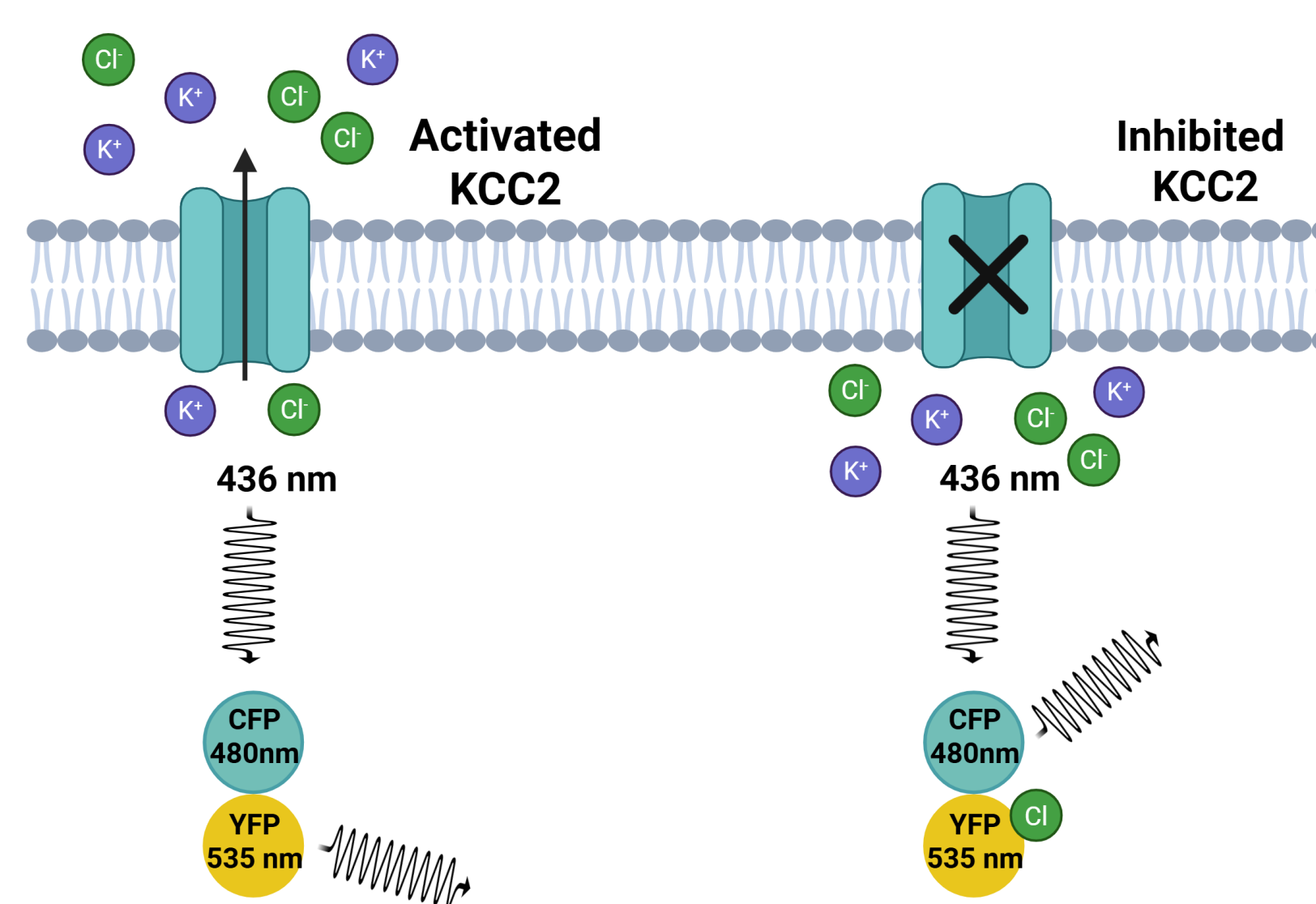
- KCC2 is the principal neuronal K⁺/Cl⁻ symporter for maintaining low intracellular Cl⁻ and enabling hyperpolarizing GABAergic signaling.
- Reduced KCC2 function is linked to epilepsy and other CNS disorders, making its enhancement a promising therapeutic strategy.
- Axxam developed two complementary HTS-grade cellular assays allowing the measurement of forward and reverse KCC2 activity.



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ASSAY PRINCIPLE

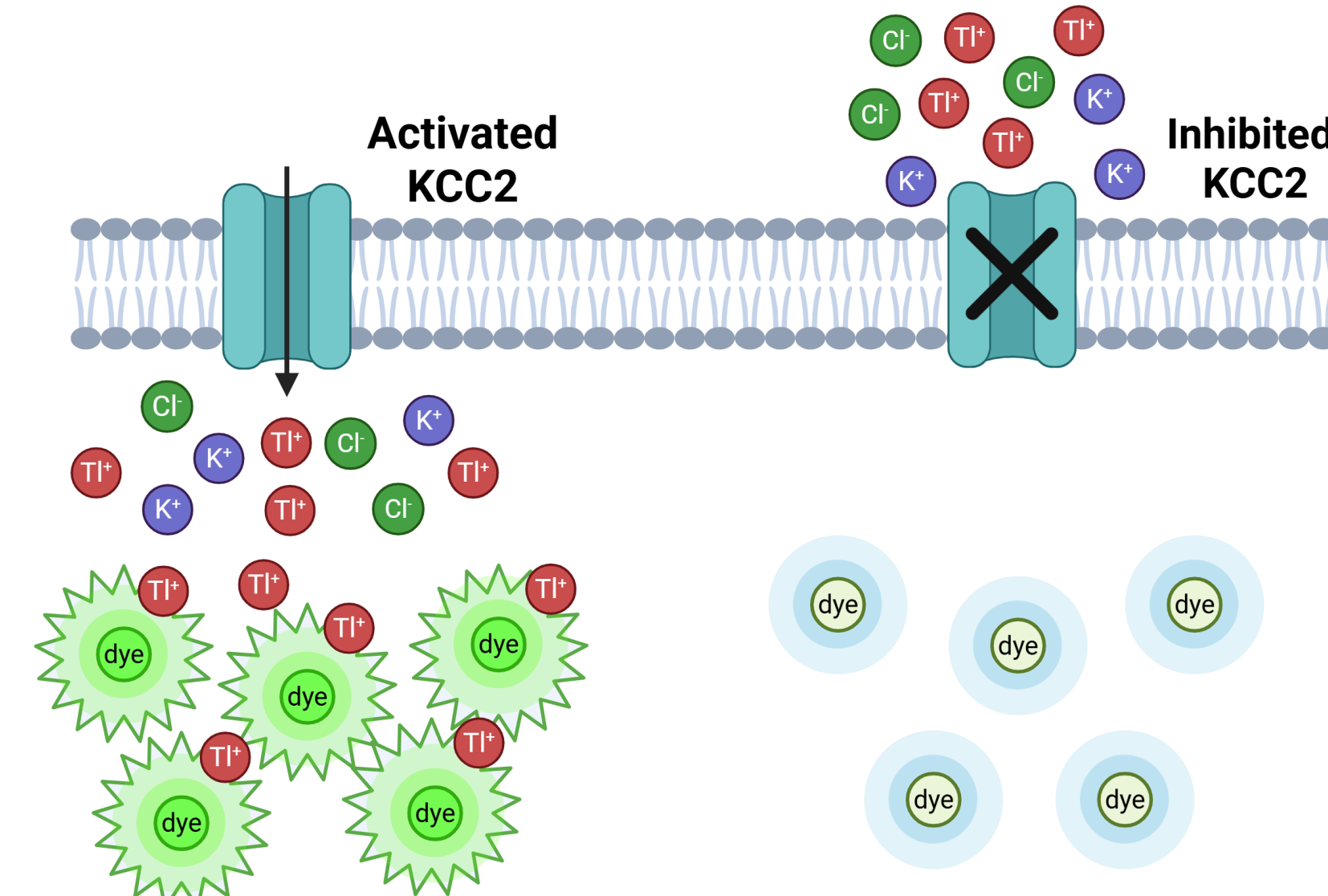
SUPERCLOMELEON ASSAY: KCC2 Forward Mode



ON SIGNAL: FRET Ratio ↑ YFP/CFP (535 nm/480 nm)
OFF SIGNAL: FRET Ratio ↓ YFP/CFP (535 nm/480 nm)

- SuperCameleon is a ratiometric CFP-YFP FRET-based biosensor in which the YFP moiety is sensitive to intracellular chloride, allowing real-time detection of Cl⁻ dynamics.
- This approach provides a sensitive and stable readout, which enables the accurate monitoring of forward KCC2 physiological transport under near-physiological conditions.

THALLIUM ASSAY: KCC2 Reverse Mode



- Thallium (Tl⁺) acts as a functional surrogate for K⁺, entering cells through KCC2 when ionic conditions drive the transporter in reverse.
- This readout allows indirect but robust monitoring of KCC2 activity.

ASSAY DEVELOPMENT

- Two inducible target-expressing cell lines were generated (corresponding mock cell lines were created in parallel as negative controls).
- Limiting dilution and functional clone pool analysis enabled selection of the best-performing clones (target cell lines).
- Target expression and assay functionality were confirmed by qPCR analysis and literature reference compounds, respectively.
- The final selected best clones were optimized for high-throughput screening (HTS) campaigns.

Stable Cell Line Generation

- Target cell lines: HEK T-REx SuperCameleon/KCC2
HEK T-REx/KCC2 (TI assay)
- Control cell lines: HEK T-REx SuperCameleon/mock
HEK T-REx/mock (TI assay)

Assay Optimization Workflow

Stable transfection and selection of stable pools

Limiting dilution and functional analysis for clone selection

Best clone characterization

•Cell density
•Induction protocol

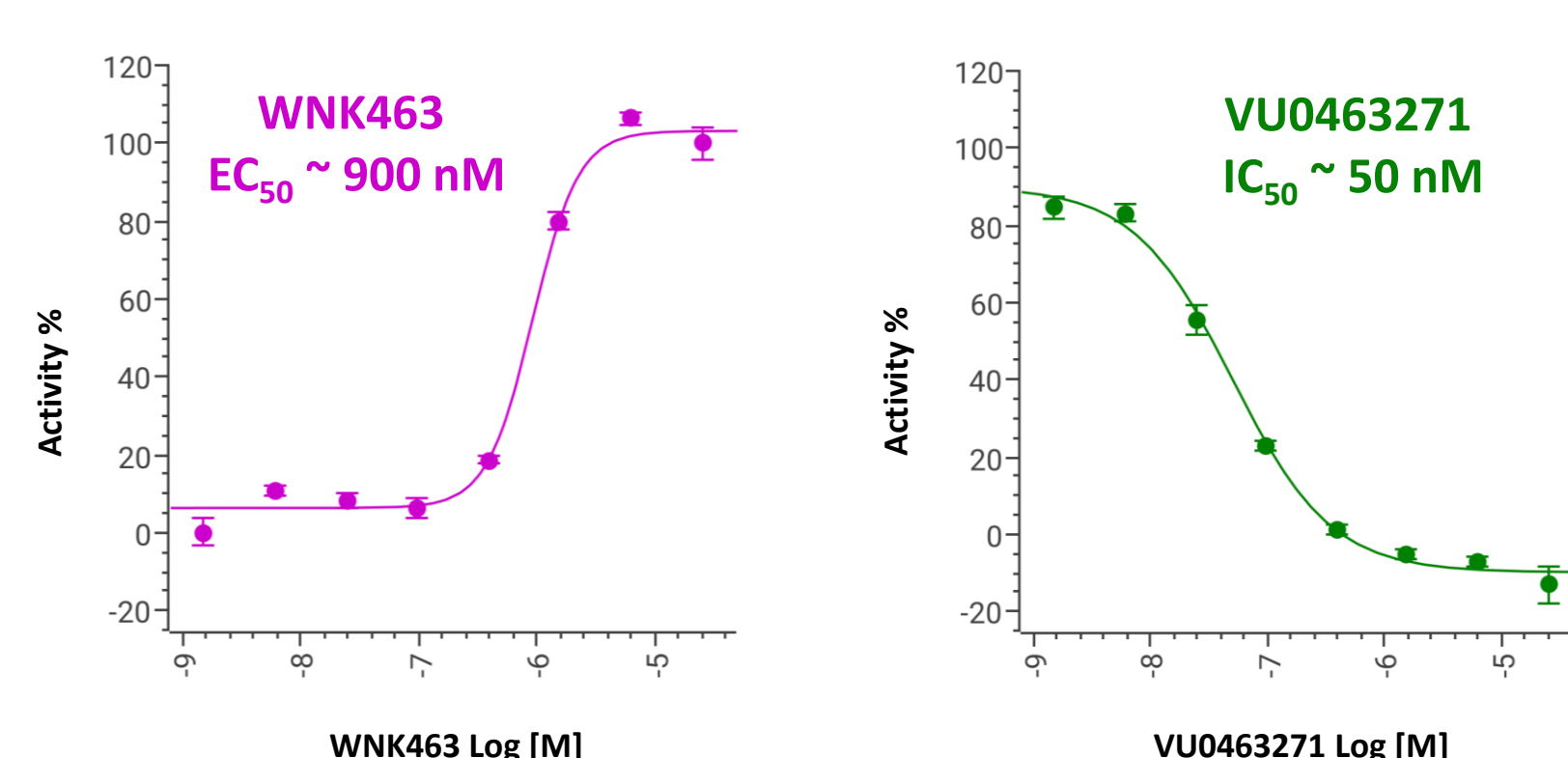
•DMSO tolerance
•Assay Robustness

Dual-assay HTS-optimized platform

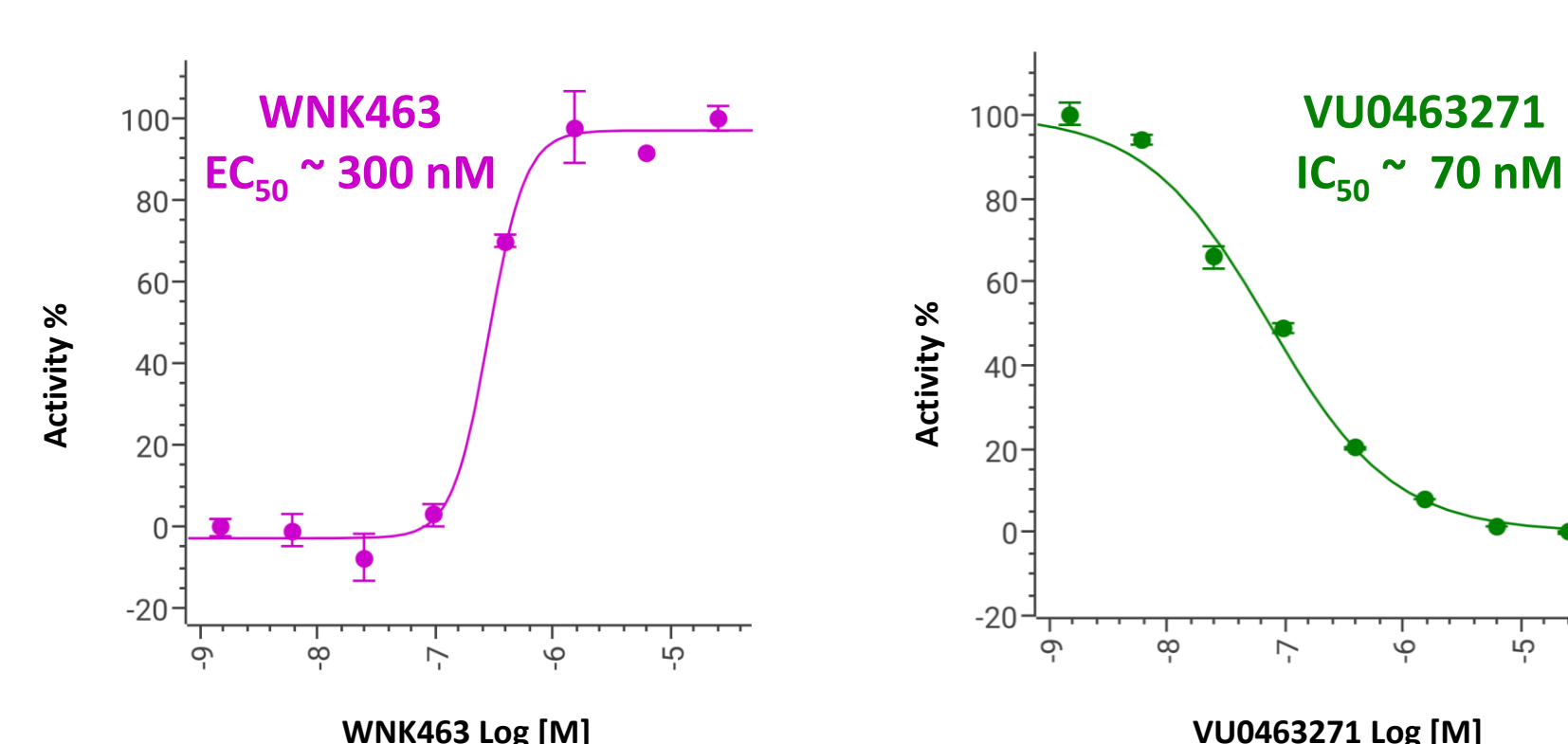
ASSAY VALIDATION

- Assay development was performed using WNK463, a pan-WNK-kinase inhibitor, as reference compound.
- Signal specificity was confirmed using VU0463271, a potent and selective inhibitor of KCC2.
- Reference compounds showed consistent results, in agreement with literature, with fully comparable outcomes across readouts.

SUPERCLOMELEON ASSAY



THALLIUM ASSAY

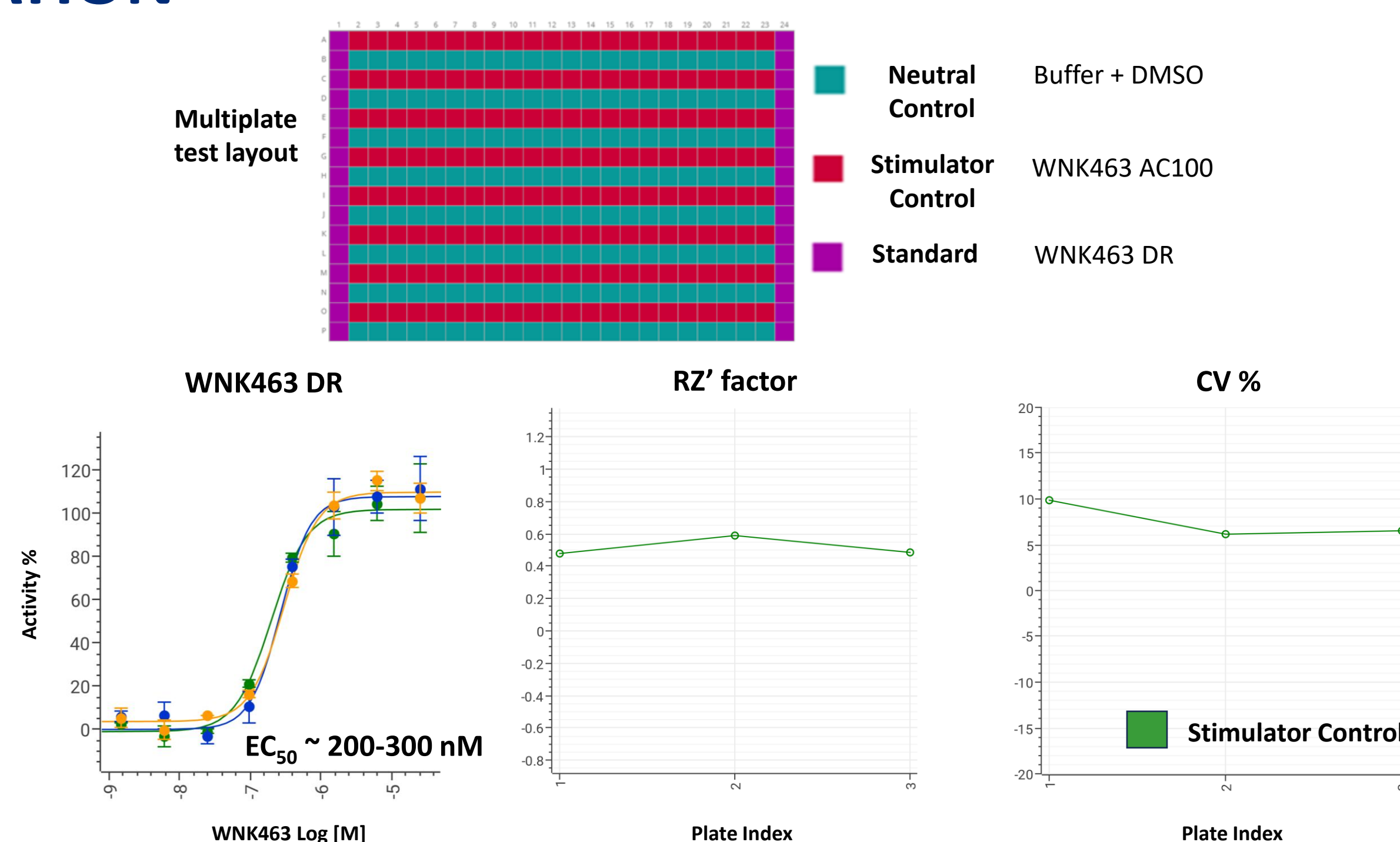


Signal specificity validated by activator and inhibitor profiles

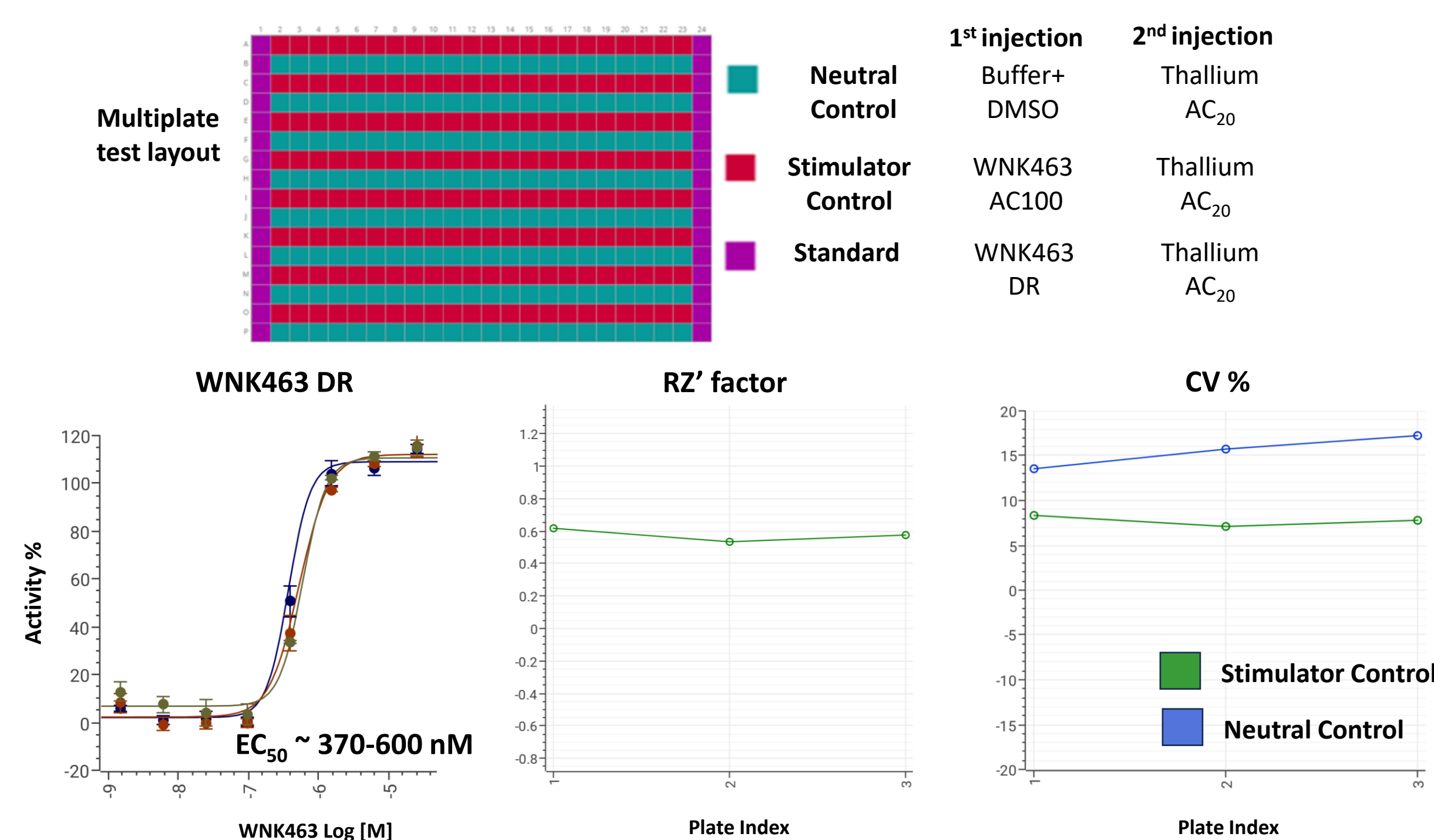
ASSAY OPTIMIZATION

- Different parameters were evaluated and optimized.
- The assays, developed in 384-well plate format, demonstrated robustness and reproducibility, thus allowing high-throughput screening to evaluate candidate potentiators for KCC2.

SUPERCLOMELEON ASSAY ROBUSTNESS



THALLIUM ASSAY ROBUSTNESS



Consistent pharmacology across plates, Robust Z' values > 0.5 and Coefficient of Variation (CV) values < 20%

CONCLUSIONS

- The present study illustrates the successful development of two complementary optimized readouts leading to an HTS-ready platform for discovering KCC2 potentiators.
- The SuperCameleon assay provides a near-native primary measurement of KCC2-mediated chloride dynamics, while the thallium assay may offer a secondary validation.
- Combined with structural insights from recent KCC2 cryo-EM studies, this dual-readout approach accelerates hit identification and can be extended to other chloride-transporting SLCs.

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