pivotpark
screeningcentre

Label-free Mass Spectrometry ultra-High-Throughput Screening

A game-changer in small molecule lead discovery.

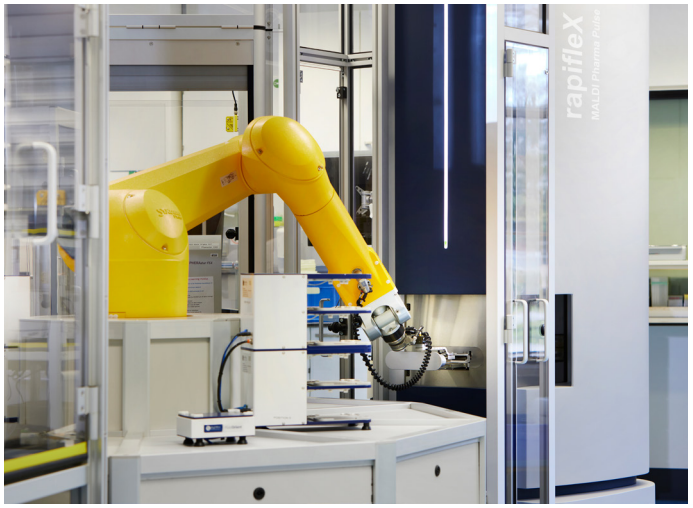
Pivot Park Screening Centre (PPSC) is the first contract research organization (CRO) that integrated the rapifleX® MALDI PharmaPulse® (Bruker AG) along with CyBio Well vario system (Analytik Jena AG) into its fully automated ultra-High Throughput Screening (uHTS) platform.
Since then, PPSC has continuously been expanding its label-free uHTS assay portfolio for various target classes. Conventional high-throughput technologies for small molecule hit discovery are often based on chemical probes, coupled secondary enzymatic reactions, and biomolecular reporters as a surrogate for assaying target activity or abundance. Caveat of these label-based methods is the risk of identifying false positives or low active confirmation hit rates. Moreover, extended efforts in development of hit triage assay cascades are required. Therefore, label-free methods in which target molecules of interest are directly quantified, are highly desirable to reduce the risk of readout technology interferences. Recent technological advances in mass spectrometry (MS) uHTS technologies, such as Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF), have paved the way for a fast adaptation of label-free readouts into large scale compound screening operations: a game-changer in small molecule lead discovery.

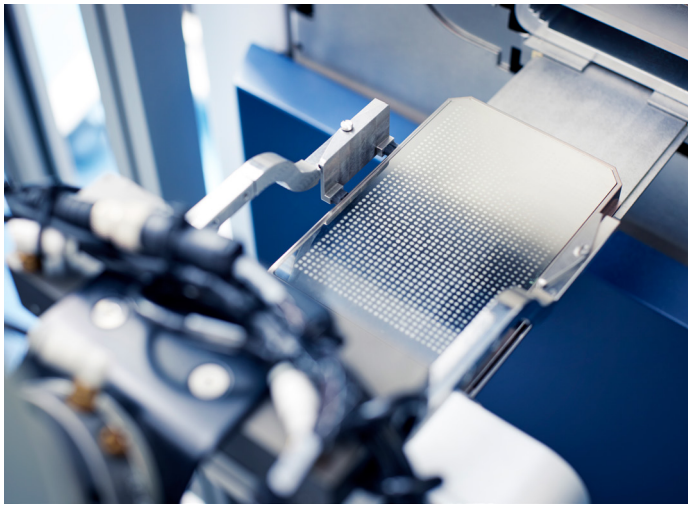
Here, we setup a showcase to illustrate the development of a high-quality and biochemically sound MALDI-TOF assay in 1536-well plate format for a well-characterized druggable target class. We carried out a fully automated label-free uHTS campaign of > 24K compounds, randomly selected from our in-house 300K+ compound collection, performed follow-up hit triage and evaluated the compound structures to provide a final hit list of 130 confirmed caspase-6 (cas-6) inhibitors.

SHOWCASE →

Panorama view of the uHTS platform at PPSC.

Close-up of the rapifleX® MALDI PharmaPulse® (Bruker AG) at PPSC.



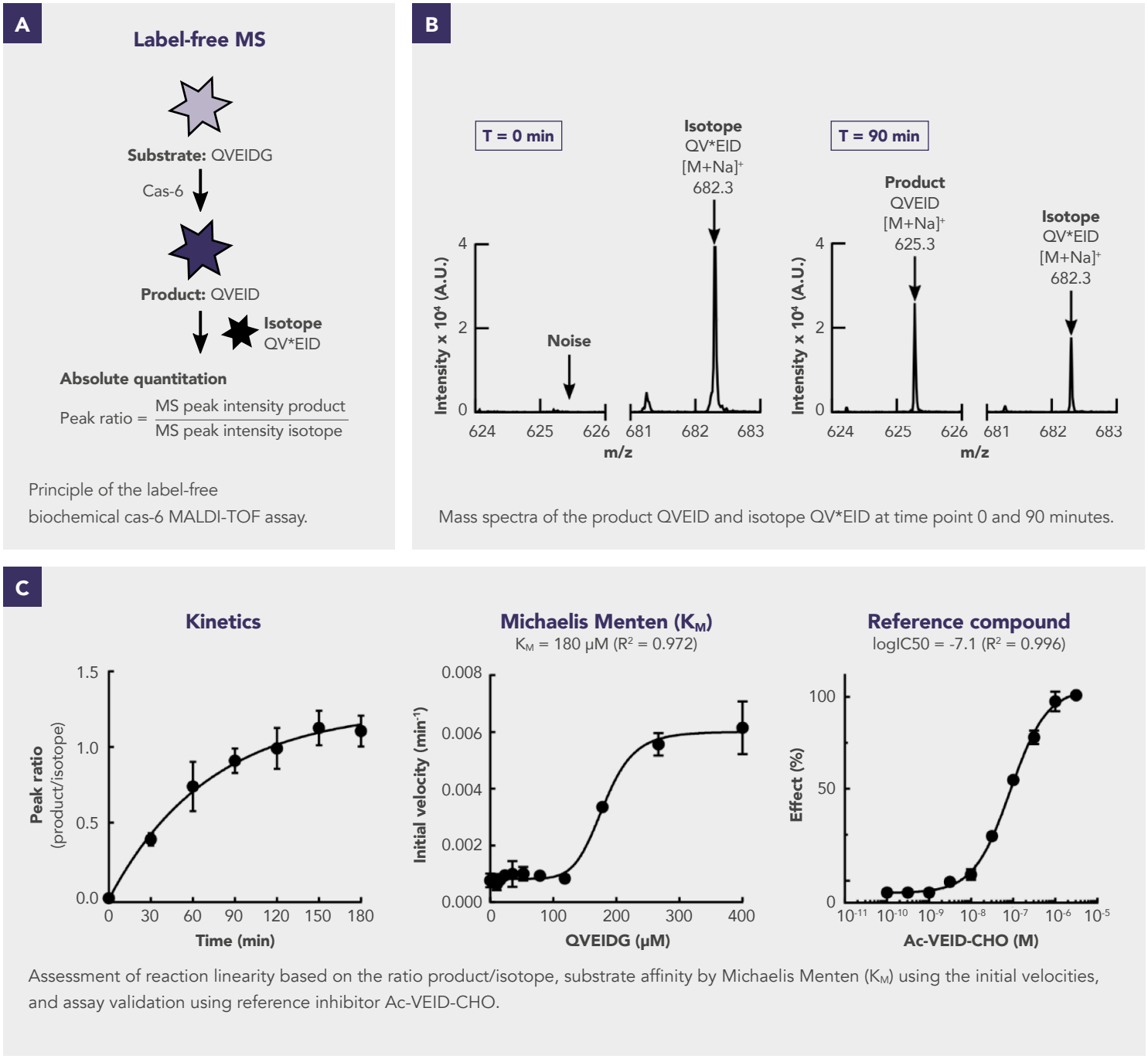


Assay development and optimization in 1536-well plate format

Cas-6 is a member of the cysteine protease family and dysregulation of this enzyme has been linked to the pathogenesis of various diseases, such as inflammatory diseases, neurological disorders, metabolic diseases, and cancer.[1-3] To this end, we designed a biochemical assay to monitor the activity of cas-6 by MALDI-TOF in 1536-well format (Figure 2). A previous reported small peptide substrate (QVEIDG), which is cleaved by cas-6 resulting in the product QVEID, was used in the assay.[4] For the absolute quantitation of the product, an isotopically labeled QVEID peptide (QV*EID) was included as internal standard. During assay development and optimization, we determined the reaction linearity and optimal substrate concentration based on Michaelis-Menten constant (K_M) to increase the likelihood of identifying inhibitors with different modes of action unbiasedly. Furthermore, the assay was validated using a previously reported covalent inhibitor Ac-VEID-CHO as reference compound.

1. Flores, J., et al., Cell Death & Differentiation (2022) 29: 657-669.
2. Zheng, M. et al., Cell (2020) 181: 674-687.
3. Ladha, S., et al., Cell Death Discovery (2018) 4:40
4. Adam, G.C., et al. Journal of Biomolecular Screening (2015) 20: 212-222.

Development, optimization, and miniaturization of a label-free MALDI-TOF-based uHTS assay for cas-6.



Principle of the label-free biochemical cas-6 MALDI-TOF assay.

Mass spectra of the product QVEID and isotope QV*EID at time point 0 and 90 minutes.

Assessment of reaction linearity based on the ratio product/isotope, substrate affinity by Michaelis Menten (K_M) using the initial velocities, and assay validation using reference inhibitor Ac-VEID-CHO.

Stable assay performance during MALDI-TOF uHTS campaign and high-quality active confirmation of primary actives.

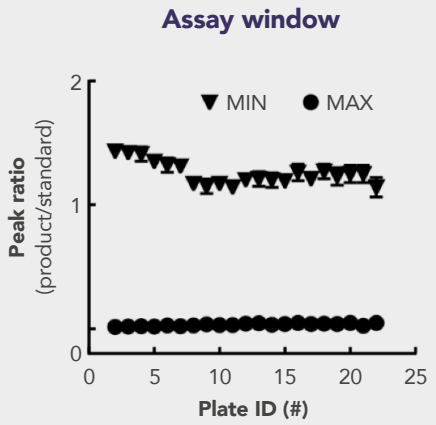
uHTS campaign and active confirmation

Upon achieving feasible and biochemically sound conditions, we optimized and miniaturized the cas-6 MALDI-TOF assay for uHTS by testing different dispensing steps, final volumes, reagent stability, inter- and intra-plate variation, DMSO tolerance, protein freeze/thaw influences, storage conditions, and readout stability. Acceptable uHTS criteria are assays with Z-prime > 0.5, signal-to-background (S/B) > 3, and a final volume per well < 8 µL in 1536-well plate format.

Once these criteria were met, we selected > 24,000 compounds randomly from PPSC's 300K+ in-house chemical diversity-based compound collection comprising high-quality drug-like compounds with > 22K unique scaffolds. The MALDI-TOF uHTS campaign was performed in single point (10 µM) with a lead time of less than a day showing a stable assay window and Z-prime. During the screening campaign, a quality control (QC) plate with a dilution series of the reference compound was tested at the beginning and at the end of the run, resulting in a stable IC50 value (data not shown).

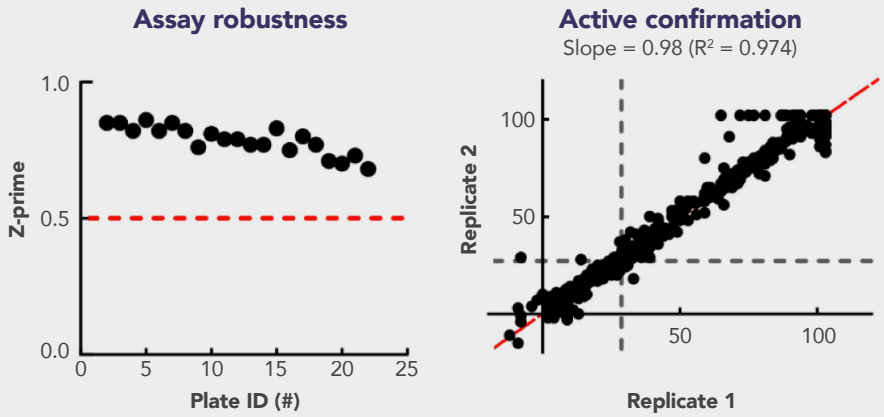
We selected 574 primary actives using the criteria Z-Score ≤ -4 and inhibitory effect ≥ 25 %. In total, 561 compounds were reordered and tested for active confirmation in duplicate. The inhibitory activity of 511 compounds from the 561 selected primary actives were confirmed with **91 % active confirmation rate and R^2 = 0.97** for the duplicates.

A



Overview of assay window and robustness based on MIN and MAX effect during uHTS campaign with an S/B (= $\frac{MIN}{MAX}$) 24 - 98, and Z-prime (=1- $\frac{3 \times (Stdev_{MIN} + Stdev_{MAX})}{MIN - MAX}$) 0.68-0.86.

B



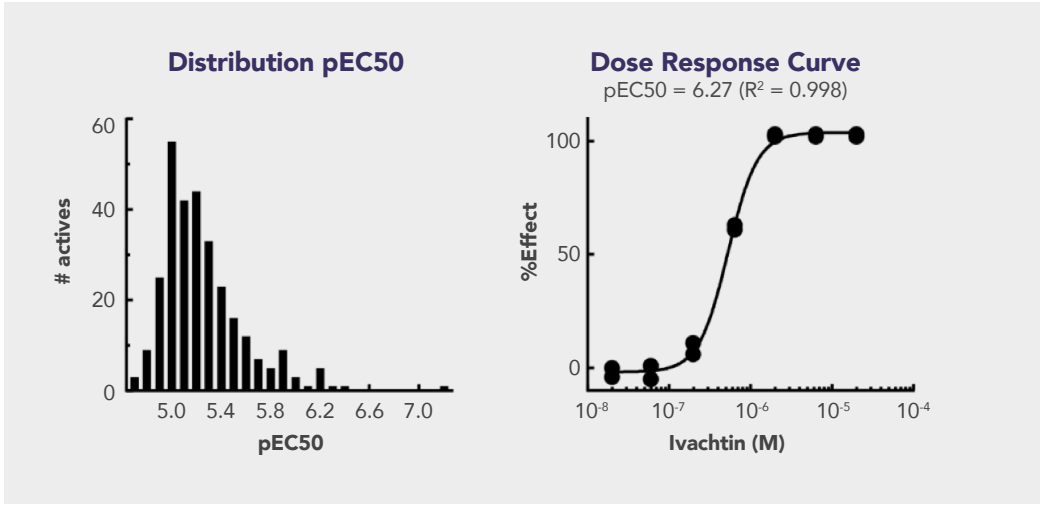
Active confirmation of 561 selected primary actives in duplicate. Actives were selected based on Z-Score (= $\frac{Signal_{SAMPLE} - MIN}{Stdev_{MIN}}$) ≤ -4 and effect (= $\frac{Signal_{SAMPLE} - MAX}{MIN - MAX}$ x 100 %) ≥ 25 %.

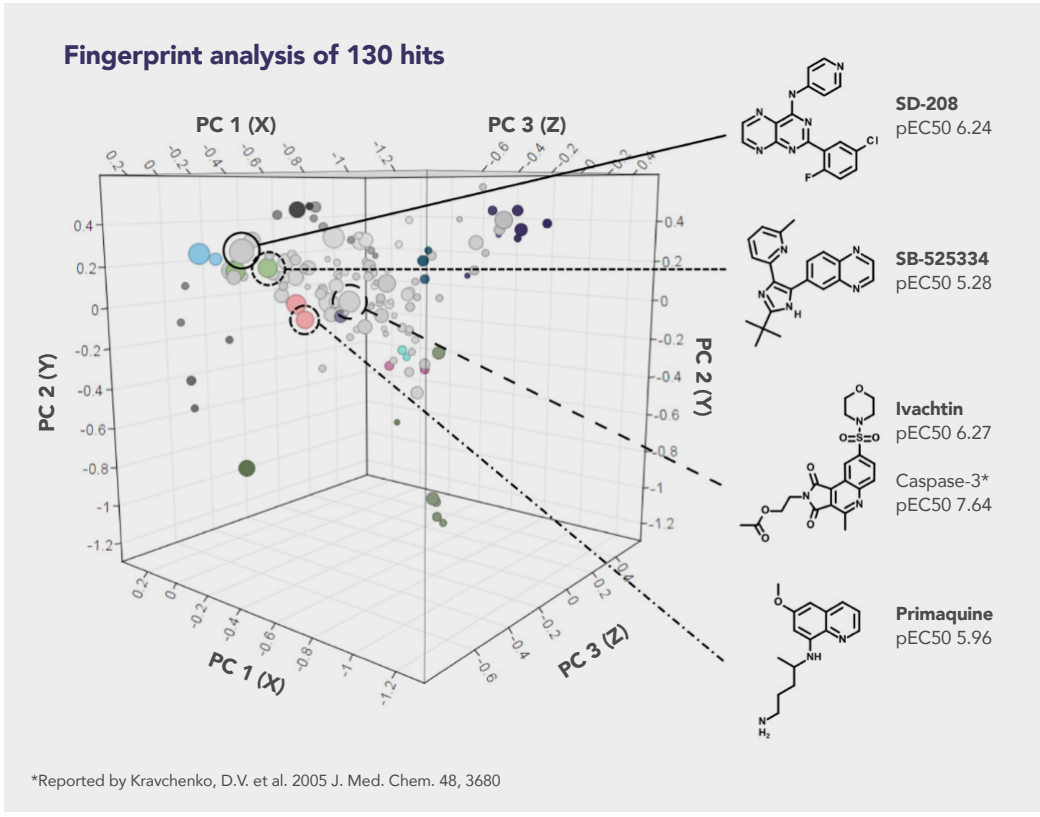
Hit confirmation and follow-up characterization

From the 511 confirmed actives, 256 compounds were prioritized for Dose-Response Curve (DRC) testing. Compounds with a pEC50 > 5.0 were selected for further triage, taking potential covalent inhibitors and clustering of actives into consideration. This resulted in a final hit list of 130 known and novel cas-6 inhibitors, 42 of which were distributed over 13 different clusters and 88 compounds were singletons. The results were visualized using Principal Component Analysis (PCA) of chemical fingerprints of the hits. Few examples of the confirmed hits are Ivachtin (a known caspase-3 inhibitor), SD-208, SB-52334 and Primaquine. Altogether, these results show that MS-based uHTS is a robust and versatile screening method for identifying drug-like chemical starting points for drug discovery.

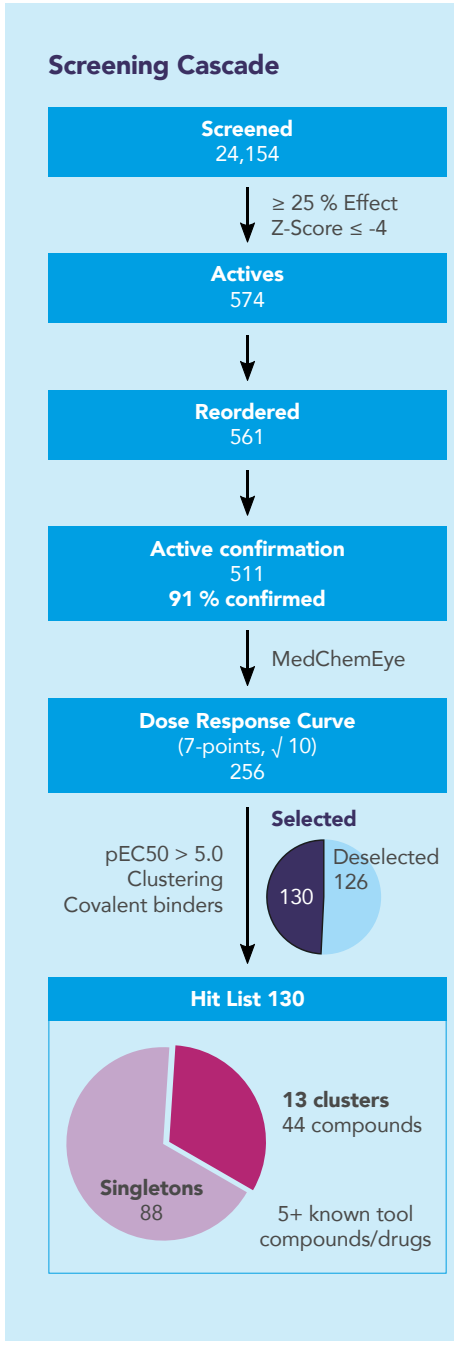
Overview of the MALDI-TOF uHTS campaign (left panel). 256 primary actives were analyzed in dose response curves, whereof a final hit list was selected based on pEC50 > 5.0, compound clustering and covalent binders. Chemical fingerprints of the final hit list resulted in 88 singletons and 13 clusters comprising 42 compounds.

Cascade of selecting primary actives and follow-up studies of dose response curve and principal component analysis.





*Reported by Kravchenko, D.V. et al. 2005 J. Med. Chem. 48, 3680

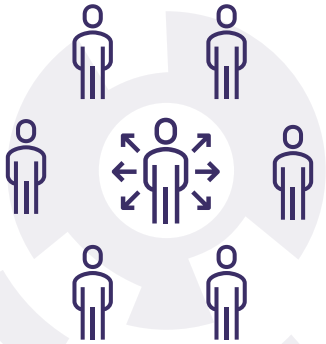


Conclusions

PPSC has

- successfully integrated MALDI-TOF for fully automated uHTS (1536-well format).
- developed a robust and biochemically sound label-free assay from scratch.
- performed an uHTS campaign using a label-free MS biochemical assay with absolute quantitation.
- generated > 24K high-quality datapoints within less than a day.
- capacity of approximately 90-100K compounds per day for MALDI-TOF uHTS.
- shown 91 % active confirmation rate with R^2 of 0.97 for the replicates.
- identified 130 hits, including 13 new clusters and 88 singletons from screening a small subset of the PPSC compound collection.

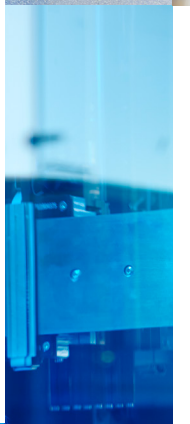
A PPSC project team is built around your needs



PPSC is a project-driven organization and as such uses leading project management tools to ensure successful delivery of project milestones. Our highly skilled scientists continuously monitor the project scope, progress, quality, timelines, budget and efficiency of the project teams.

Our devoted project team communicates with the client about progress, risks and deviations during the whole project cascade.

Together we accelerate drug discovery for a healthier society



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How can we help you?

Are you looking for ways to accelerate your drug discovery? Contact us to explore how we can improve global health together.

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We help you to accelerate drug discovery