REACTION BIOLOGY

Biophysical Assays

- Surface Plasmon Resonance
- Thermal Shift Assay
- Isothermal Titration Calorimetry
- Microscale Thermophoresis

Let’s discover together.

Biophysical Assays

Biophysical methods enable understanding of the drug-target interaction on a molecular level. The residence time, mechanism of action and kinetics are crucial for the therapeutic action of a drug and essential for the selection of analytes with the most promising properties for the next stage of drug discovery. Our team of biophysics experts, together with an array of cutting edge instrumentation, is available for hit identification, hit-to-lead and lead characterization activities tailored to the needs of your research project.

Overview of the Biophysical Assay Platform

Biophysical assay techniques have become essential tools in today's drug discovery activities. Below is an overview of the four biophysical methods that are available at Reaction Biology including surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), microscale thermophoresis (MST) and thermal shift assay (TSA).

	Surface Plasmon Resonance (SPR)	**Isothermal Titration Calorimetry (ITC)**
Physical Principle	Optical measurement that detects molecular mass changes of the target upon analyte binding	Measures the heat released or absorbed during analyte binding to a target
Binding Affinity Determination	√	√
Parameters Measured	Dissociation constant (K_D) Association rate constant (K_{on}) Dissociation rate constant (K_{off})	Dissociation constant (K_D) Binding enthalphy (ΔH) Binding stoichiometry
Capacity	Medium to high throughput	Low throughput
Sample Requirements	Purified target and agents required	Purified target and agents required
Dynamic Ranges	pM to mM	pM to mM
Advantages	Provides kinetic parameters (on/off rates) and is measured in real-time	Provides thermodynamic parameters and binding stoichiometry
Immobilization	Target immobilized to sensor chip	In solution
Labeling	Label-free	Label-free

These techniques provide the foundation for both small molecule and antibody drug discovery and a path toward understanding binding kinetics and mechanisms of action as crucial factors for efficacy and selectivity.

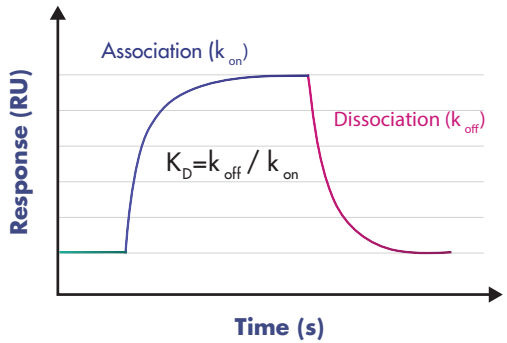
Reaction biology offers off-the-shelf assays, custom assay development and optimization as well as integrated projects in close interaction with our team of biophysical assay experts.

Microscale Thermophoresis (MST)	**Thermal Shift Assay (TSA)**
Measures the changes in the motion of target molecules along microscopic temperature gradients upon analyte binding	Monitors the shift in melting temperature of a target upon analyte binding
√	X
Dissociation constant (K_D)	Shift in melting temperature (ΔTm)
Medium throughput	High throughput
Purified target and agents required in most cases	Purified target and agents required
pM to mM	N/A
Wide range of targets including small molecules and large macromolecular complexes	Fast and inexpensive
In solution	In solution
Fluorescent labeling of the target required	Label-free

SPR – Surface Plasmon Resonance

SPR is a highly sensitive technique for accurate measurement of the interactions of two biomolecules with respect to binding kinetics and affinity as well as binding specificity. SPR is suited for high-throughput screening.

- Label-free and real-time characterization of analyte-target interaction
- Suitable to advance any analyte including fragments, antibodies, peptides, nucleic acids against any target class including enzymes and non-active proteins
- Deliverables: binding constant K_D (binding affinity), association K_{on} and dissociation rate constants K_{off}.

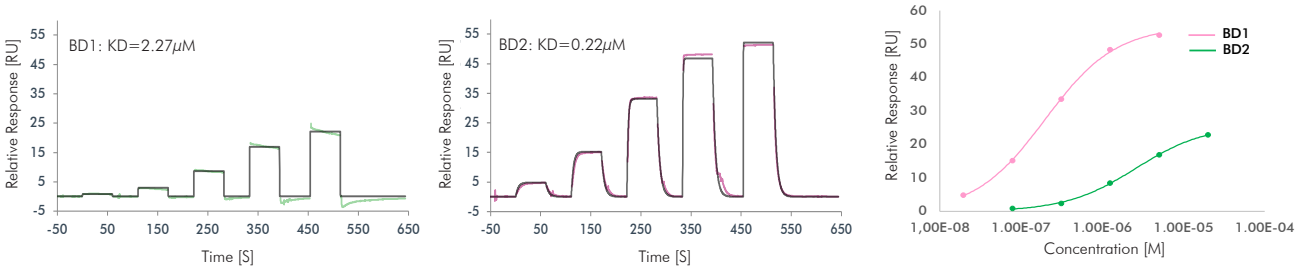


Example of the kinetic profile of an analyte-target binding reaction.
SPR detects changes in the refractive index at the surface of a sensor chip as a result of molecular mass changes of a target upon binding of the analyte.

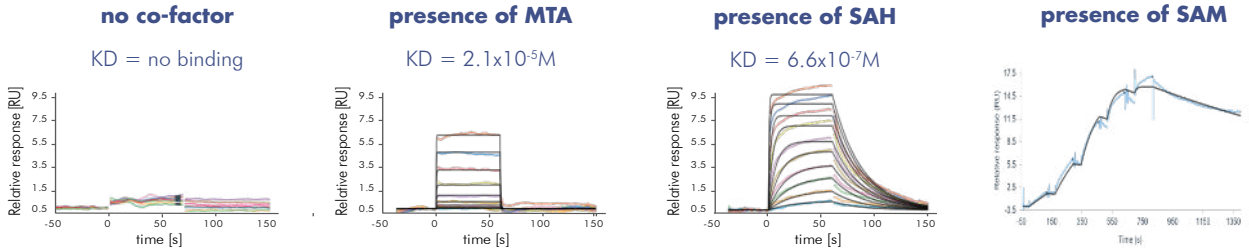
The target is immobilized to the surface of the sensor chip. During the association phase, the analyte flows over the surface and binding to the target is monitored until a steady state is reached. The flow then switches to running buffer and the dissociation of the analyte from the target is monitored.

Instruments

Reaction Biology is equipped with two state-of-the art Biacore 8K units allowing high- throughput screening with 8 channels (4600 compounds per day) with high sensitivity. In addition, the Biacore T200 unit is a versatile system for high-quality characterization of molecular interactions.



Example of binding affinity determination of an inhibitor to two bromodomains. The BD1 or BD2 domain of BRD4 were immobilized to the sensor chip surface. The analyte was applied to the chip surface in increasing concentrations approaching saturation. Right graph: The relative response at equilibrium for each dose was plotted against the analyte concentration to determine the equilibrium dissociation constant, KD. The analyte is 10-times more selective for BD2 than for BD1.



Example of a co-factor analysis by SPR. EPZ015666 is a substrate competitive inhibitor that binds to its target enzyme, PRMT5/MEP50, only in the presence of SAM or SAM analogues such as MTA and SAH. No dose-dependent responses were observed for analyte binding to apo protein (left figure). The binding to MTA-bound target is relatively weak (KD~20μM) with fast kinetics (on/off). The binding affinity increased by ~10-fold for SAH-bound target (KD~2μM). While the on-rates are similar for the MTA- and SAH-bound conditions, the off-rates are approximately 100X slower. The highest affinity (KD~3nM) and slowest off-rate (100x less than SAH-bound) was observed for analyte binding to the SAM-bound target. Single-cycle kinetics, that do not require a return to baseline in between doses, was used due to the slow off-rate observed for this condition. A slower off-rate indicates longer occupancy of the analyte on the target.

The analyte was tested with 7 concentrations depicted in different colors.

Target	ka1 (1/Ms)	kd1 (1/s)	KD (M)
Apo PRMT5	Minimal signal changes/binding		
MTA-Bound PRMT5	6.46e+4	1.03e0	1.60e-5
SAH-Bound PRMT5	3.46e+4	3.64e-2	1.05e-6
SAM-Bound PRMT5	1.55e+5	4.76e-4	3.07e-9

SPR – a versatile tool to address many challenges	
Target is not an enzyme	SPR measures the direct binding between analyte and target which is why the target does not need to be an enzyme and no substrate is needed.
Unknown substrate	
Co-factor/competition studies	The impact of various co-factors on the analyte-target interaction can be tested.
Fragment-based screening	Low molecular mass fragment compounds (100–300 Da) tend to demonstrate low binding affinity; thus, the compounds require screening at a high concentration. This is better tolerated in the SPR platform than in many biochemical assays.
Antibody screening/ characterization	SPR can be used for antibody affinity determination, determination of kinetic parameters, epitope mapping, binding specificity, and cross-reactivity.
SAR studies	The kinetics of drug binding and unbinding, especially the residence time, play a crucial role for a drug's in vivo efficacy. SPR can rank the kinetic selectivity of drug analogues for selection of the best drug candidates.
High-information content	Combining kinetic information with affinity and potency data early in the drug discovery process ensures that promising compounds are not being discarded.
Elimination of promiscous binders	Promiscous binders, which appear as false positives in biochemical inhibitor assays, can be identified by SPR when used as the secondary screening technology.
Plasma protein binding	SPR-based assays are sensitive high-throughout options to accurately measure plasma protein binding of analytes.
Unknown agent concentration	SPR can quantitate analytes in solution.

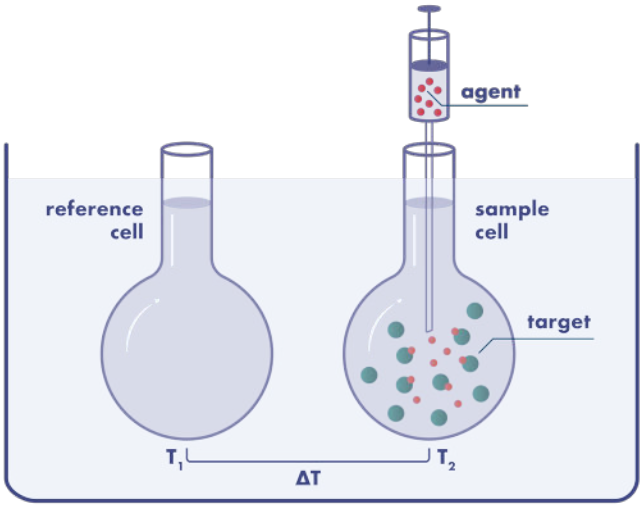
Example SPR workflow of a customized analyte-target binding study:

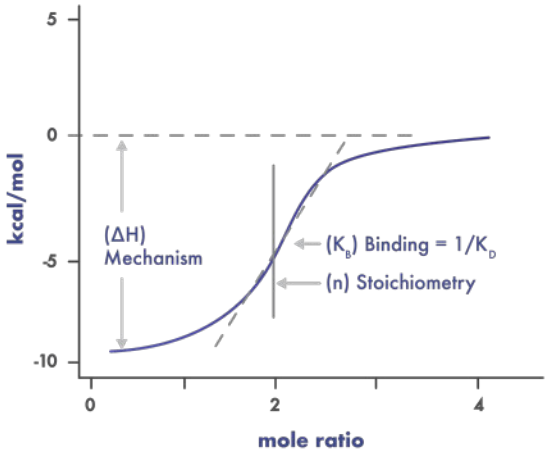
- Sensor chip preparation: Attaching the target to the sensorchip
  - Determination the best method for immobilization (Covalent coupling vs. attachment via specific tag)
  - Optimizing immobilization levels to observe analyte binding based on molecular weights of target and analyte
- Assay validation: Testing the behavior of the target on the sensor chip
  - Kinetics/affinity determination of a positive control and comparison with known KD or IC50 value
  - Optimization of buffer conditions (ex: pH, type of detergent, amount of DMSO…)
  - Determination of regeneration conditions, if necessary
- Assay reproducibility: Testing if the optimized conditions are reproducible
  - Consistency of the immobilization level from sensor chip to sensor chip
  - Consistency/reproducibility of the binding behavior of the reference compound
- Protein activity and stability on the sensor chip
  - Estimating the amount of active protein on the sensor chip based on reference compound responses
  - Measuring the control over time to determine the usable life-time of the sensor chip (levels of protein activity loss over time)
- Analyte testing
  - The binding of the test analyte(s) are measured using the optimized conditions
  - For kinetics/affinity determination either a 10 concentration multi-cycle or 5 concentration single-cycle measurement will be used (dependent upon the off-rates and regeneration conditions necessary)
  - For screening studies, a single concentration measurement can be used for analyte ranking

ITC – Isothermal Titration Calorimetry

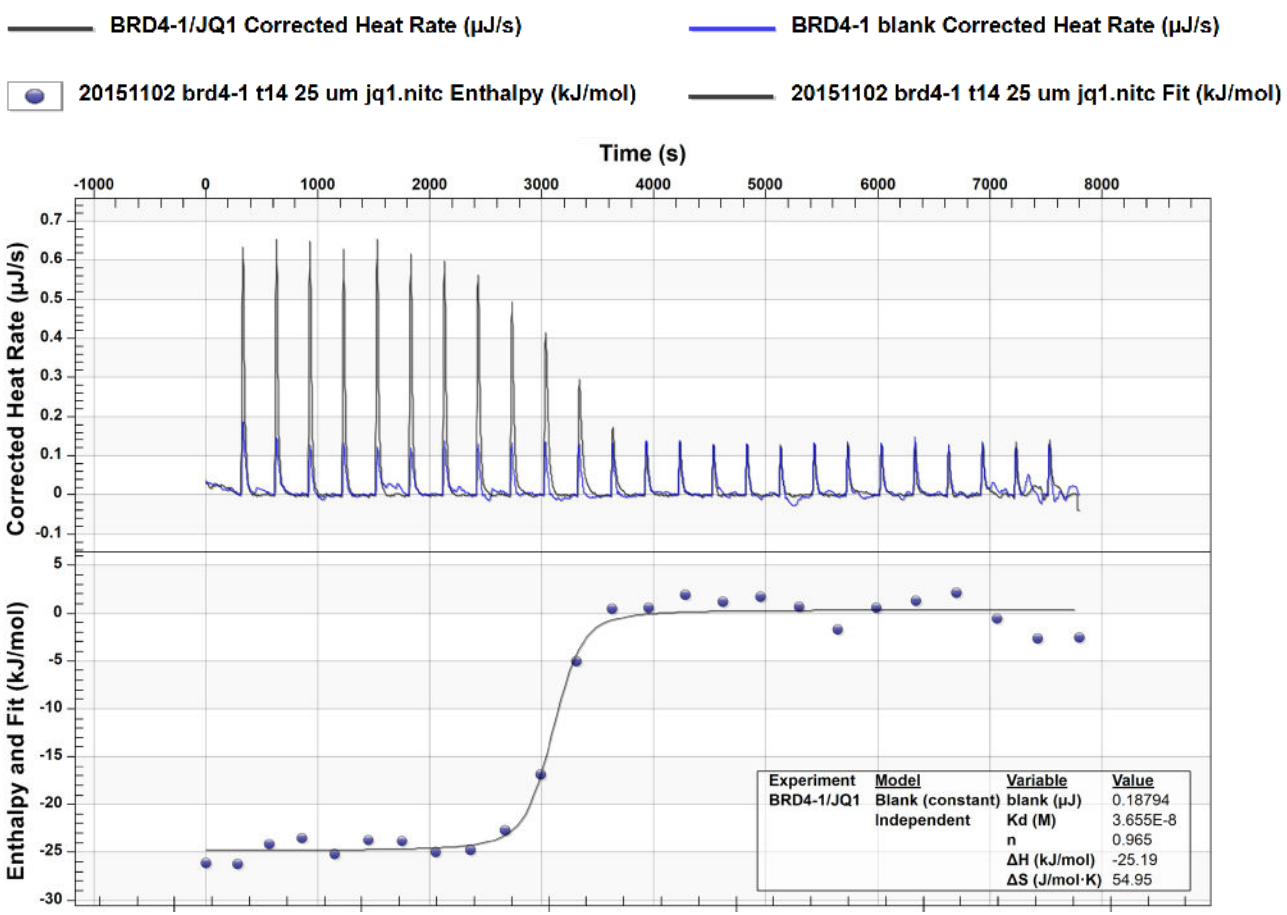
ITC measures the heat released or absorbed as the result of an interaction such as ligand binding to a target. This technique is suitable as secondary screen yielding not only the binding affinity but also the thermodynamic profile of a ligand-target interaction giving insight into the structure-function relationship on the molecular level.

- Label-free technique avoiding any influences of labels or immobilization on the binding reaction
- Deliverables: Binding stoichiometry, binding enthalpy (ΔH), entropy (ΔS) and dissociation constant K_D (binding affinity)





Assay principle. Via an injection syringe a solution with the ligand molecules is gradually titrated into a solution with target molecules in a stable temperature environment. The heat absorbed or generated during the binding reaction is measured and allows calculation of various binding parameters.



Example of evaluation of BRD4-1 binding to JQ1. Upper image: Overlay of titration spectrograms of BRD4-1 protein vs. JQ1 (black) and buffer (blue). Lower image: integrated data for BRD4-1/JQ1 binding was analyzed using one site binding model. Best fit parameters are shown in the box. JQ1 shows binding to BRD4-1 with an estimated KD of 36 nM.

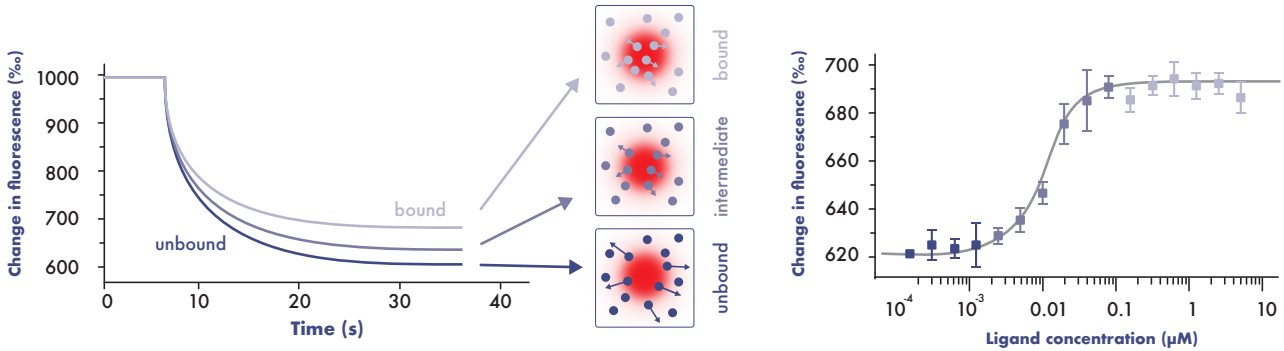
Instrument

The low volume nano ITC (TA instruments) is highly sensitive and uses less sample than the standard volume ITC instrument. The cell is constructed of inert gold that has high thermal conductivity and is compatible with harsh cleaning protocols. Cylindrical cell geometry maximizes stirring efficiency and eliminates dead zones and bubbles.

MST – Microscale Thermophoresis

MST measures the motion of molecules along microscopic temperature gradients that changes upon ligand binding

- MST requires low amounts of pure analyte sample and may be compatible with non-purified protein samples
- Compatible with most buffers.
- Binding between any types of biomolecules can be investigated including small-molecules, multi-protein complexes, DNA, liposomes, nano-particles and more
- The method requires samples that are fluorescent or have a fluorescent dye attached
- Deliverable: dissociation constant K_D (binding affinity)



Assay principle. When performing an MST experiment, a microscopic temperature gradient is induced by an infra-red laser and the movement of molecules away from the heated area is monitored. The movement varies depending on whether a ligand is bound to the molecule and the difference in motion can be used to calculate binding affinity.

Instrument

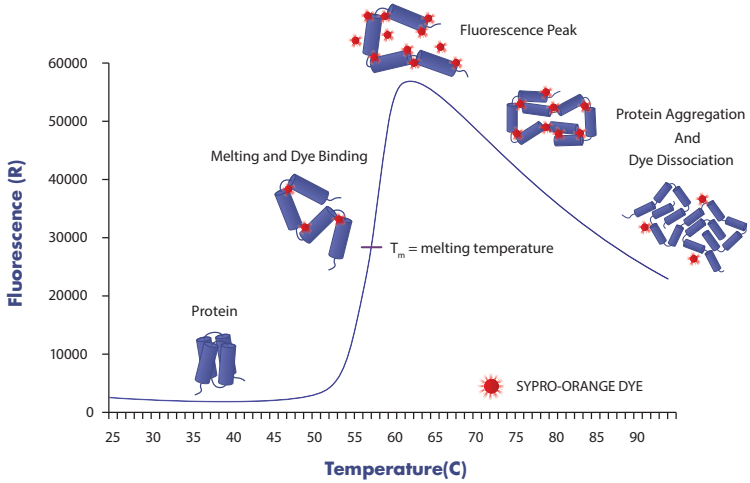
Monolith NT.115 pico (NanoTemper)

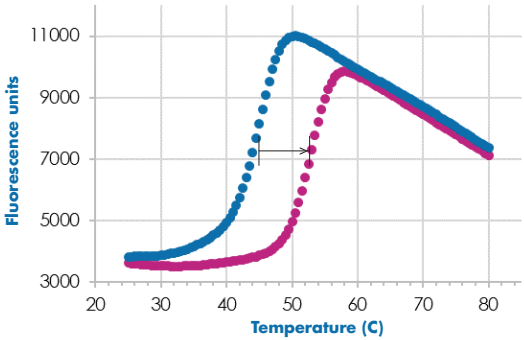
TSA – Thermal Shift Assay

Thermal shift assays, also referred to as differential scanning fluorimetry, measure the thermal stability of a target protein and the increase in protein melting temperature upon the binding of a stabilizing agent. Reaction Biology performs thermal shift assays using a qPCR machine for high-throughput data acquisition in the 384 well format – in a matter of hours.

- Useful to identify ligands, buffer conditions and cofactors
- Fast setup and minimal establishment requirements
- Deliverable: melting temperature shift (ΔTm)

Assay principle. TSA measures the melting temperature of a protein (Tm) which is the temperature at which there is 50% denaturation. Protein denaturation is monitored via increase in fluorescence of SYPRO Orange dye, that binds to hydrophobic residues that get exposed as the target protein unfolds. SYPRO Orange dye has an excitation/emission wavelength profile compatible with qPCR machines.





Example of thermal shift assay of cGAS in presence or absence of 10 μM PF-06928215. Thermal denaturation of cGAS is detected by binding and increased fluorescence of the dye SYPRO Orange. Addition of the inhibitor PF-06928215 stabilizes the the protein and increases the melting temperature from 45 to 53 degrees Celsius.

— DMSO

— PF-06928215

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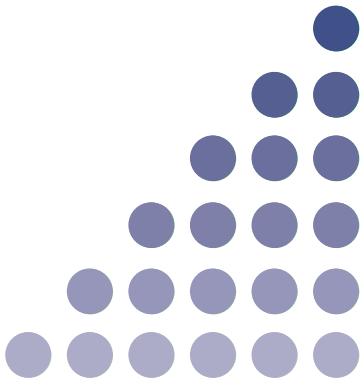

Let's Discover Together.

Recombinant Proteins

- Kinase proteins
- Epigenetic proteins
- Substrates
- Custom-tailored protein production

Target-Specific Assays

- Biochemical and cell-based assays
- Enzymatic activity testing
- Protein: Protein Interaction assays
- Receptor Biology

Cellular Oncology

- 2D and 3D proliferation assays
- Drug combination screening
- Invasion and migration assays
- Angiogenesis assay

Biophysical Assays

- Surface Plasmon Resonance
- Thermal Shift Assay
- Isothermal Titration Calorimetry
- Microscale Thermophoresis

In Vivo Pharmacology

- In Vivo Hollow Fiber Model
- Xenograft models
- Orthotopic models
- Metastasis models

Safety & Adme-Tox

- Cardiac Safety Panel
- CYP inhibition
- PK/PD studies
- In Vitro Safety Panel

Integrated Drug Discovery

- Target research
- Hit identification
- Hit-to-Lead
- Lead optimization

Biomarker Discovery

- Genomic biomarkers
- Protein biomarkers
- Immunophenotyping

Immuno-Oncology

- In Vitro Killing Assays
- Syngeneic Mouse Models
- Propriatary Tumor Models
- Immunophenotyping

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