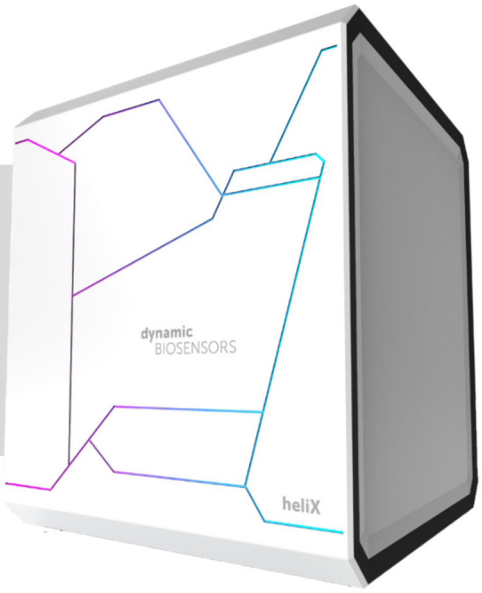
TUTORIAL
DK-YS-1

dynamic BIOSENSORS

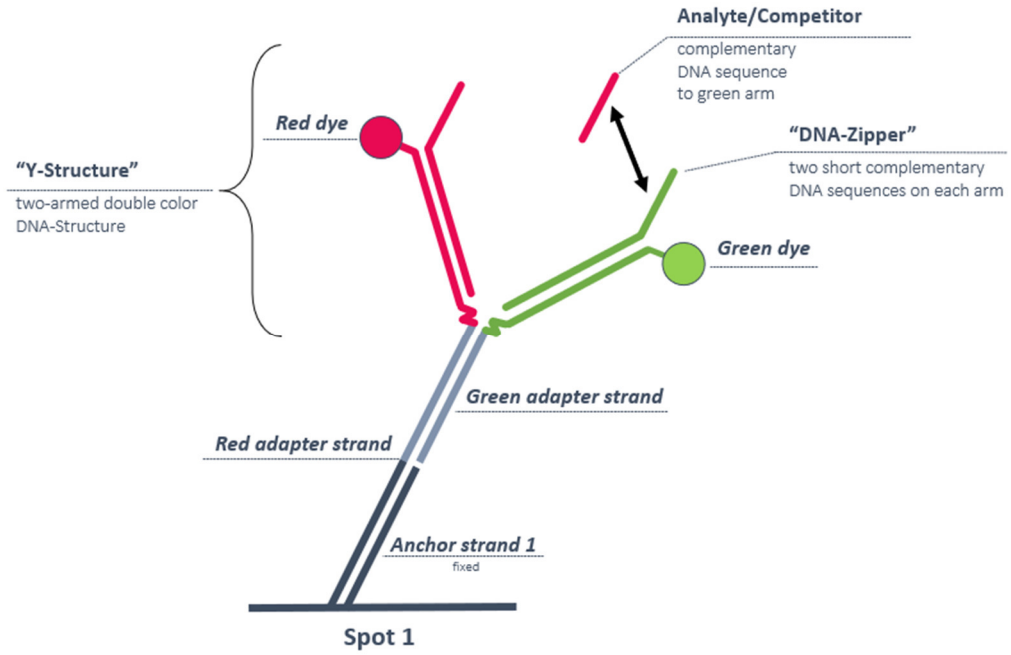
heliX® Y-Structure FRET Kinetics Tutorial

Protocol for conducting a kinetic **FRET** experiment

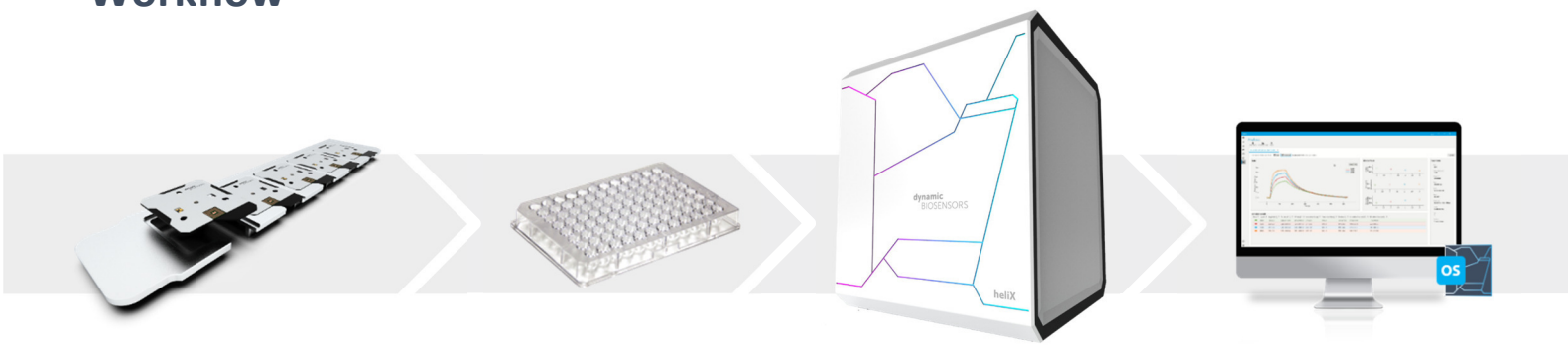


Key Features

- Replacement binding kinetics experiment of a DNA-DNA interaction:
  Analyte (5-, 6-, 7-, 8- and 9-mer oligonucleotide competitor) binding to a ligand (9-mer DNA overhang at the **green** arm of the **Y-Structure**)
- **FRET** signal read-out
- Assay setup and data analysis with **heliOS** software.



Workflow

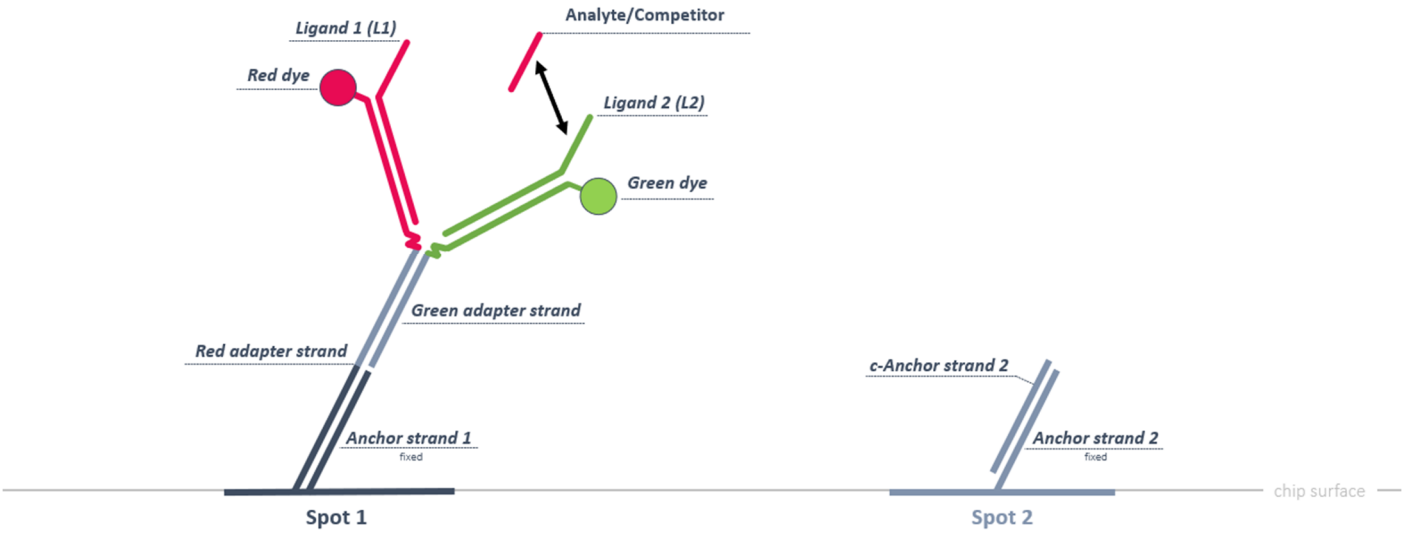


Load **heliX**® adapter biochip

Load ready-to-use 96-well plate

Run Y-Structure FRET Kinetics Tutorial

Interpret data with **heliOS** automatic analysis



Product Description

Order Number	**DK-YS-1**
Measurement Time	50 min
heliOS Software Version	1.3.0

The purpose of this **Y-Structure FRET Kinetics Tutorial** is to demonstrate a successful FRET transfer on the two-armed Y-Structure. The opening and closing of the Y-Structure can be visualized in real-time, when a FRET signal is established between the green (donor) fluorophore arm and the red (acceptor) fluorophore arm of the Y-structure in a closed formation, and subsequently lost upon opening of the structure.

In this tutorial, the competition binding kinetics of a series of DNA analyte competitors (5-, 6-, 7-, 8- and 9-mer oligonucleotide) to their matching ligand on the Y-Structure is measured using FRET as a signal read-out. To create a competition scenario, the Y-Structure is modified with 9-mer overhangs (Zipper) on the red and the green arm which are complementary to one other. The 9-mer overhang at the green arm serves as the ligand for this competition assay. At the start of the assay, the Y-Structure is in a closed formation with the Zipper-overhangs on each arm mainly hybridized, maintaining a constant FRET from the green to the red dye while in close proximity. When a competitor oligonucleotide is added during the association phase it competes with the red Zipper-overhang by binding to the green zipper(ligand) thereby shifting the equilibrium during its association phase to the open state, abrogating the FRET. In the following dissociation phase this process is reversed, the equilibrium is shifted again to a closed Y-Structure and the FRET is re-established.

This DNA-based Y-Structure assay set-up mimics an inhibition assay of a protein-protein interaction (e.g. dimerization) by a competitor/drug, with an ultimate end application of compound hit screening and target validation.
This kit is suitable for any **heliX**® device and contains material for 3 FRET binding kinetics experiments in a ready-to-use 96-well plate. One experiment includes a ligand solution for surface functionalization and five different analyte solutions (5-, 6-, 7-, 8- and 9-mer oligonucleotide) in the same concentration.

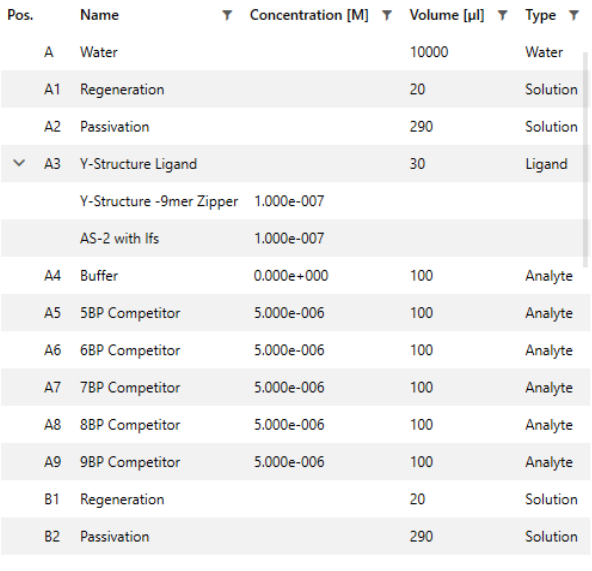
The content of the kit is summarized in Table 1. Additional required materials for this experiment are listed in Table 2.

Details about the experimental procedure are presented below:
First, the sensor surfaces of an Adapter Biochip are regenerated and functionalized:

- **Spot 1** with ***Adapter 1*** **- Ra** and **Ga** prehybridized with ***Ligand strands*** carrying the Y-Structure with the ligand (DNA-overhang); this is the measurement spot.
- **Spot 2** with ***Adapter 2*** **- lfs** prehybridized with the ***Ligand-free-strand***; this spot is not used for measurements in this tutorial.

In the second step the FRET-kinetics measurement is performed: a buffer blank is injected followed by the injection of a series of five analyte concentrations. Dissociation is performed after each concentration. The same ligand (DNA-overhang) remains on the surface for all analyte injections as the analyte dissociates completely from the ligand. The analyte (5- to 9-mer) will only bind to its ligand (DNA-overhang) on **Spot 1**.

TABLE 1 | Contents and Storage Information.

Material	Cap	Amount	Storage
Ready-to-use 96-well plate 	Sealing foil	1	-20°C

Pos.	Name	Concentration [M]	Volume [µl]	Type
A	Water		10000	Water
A1	Regeneration		20	Solution
A2	Passivation		290	Solution
A3	Y-Structure Ligand		30	Ligand
	Y-Structure -9mer Zipper	1.000e-007		
	AS-2 with Ifs	1.000e-007		
A4	Buffer	0.000e+000	100	Analyte
A5	5BP Competitor	5.000e-006	100	Analyte
A6	6BP Competitor	5.000e-006	100	Analyte
A7	7BP Competitor	5.000e-006	100	Analyte
A8	8BP Competitor	5.000e-006	100	Analyte
A9	9BP Competitor	5.000e-006	100	Analyte
B1	Regeneration		20	Solution
B2	Passivation		290	Solution

The well plate contains all required reagents for 3 runs.
Three rows (A, B and C) for each run.

TABLE 2 | Additional Material.

Material	Cap	Amount	Storage	Comment
ADP-48-2-0	—	1	2-8°C	Biochip
PE140	transparent	> 100 mL	2-8°C	Running Buffer
DI water	none	10 mL	2-8°C	—

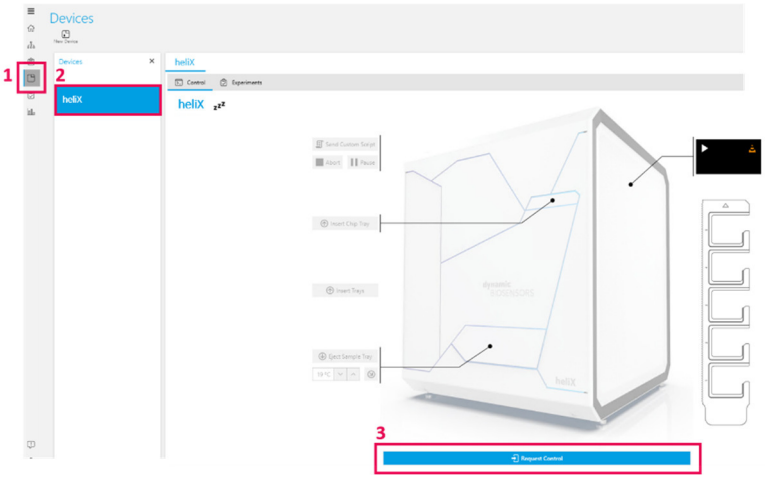
Setting up the heliX® Instrument

Buffer and Sample Preparation

Before getting started, transfer all required samples from the freezer to the fridge to slowly thaw the samples. Attach a large buffer bottle with enough PE140 running buffer (at least 100 mL) to the tubings in the buffer compartment of the **heliX®** device. Make sure that all three tubings are inside the buffer and reach the bottom of the buffer bottle and that the waste compartment is empty. Fill one 10 mL vial with deionized water. This vial without a cap will be placed on the sample tray in a subsequent step.

Instrument Preparation

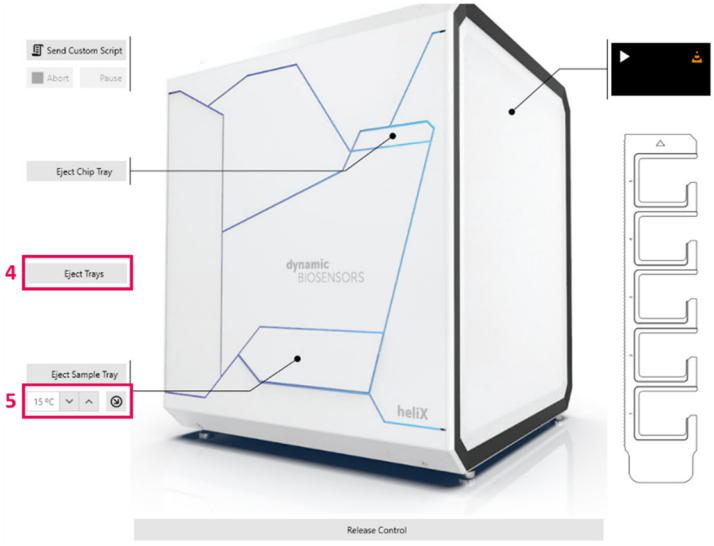
Start the latest **heliOS** software version.



(1) Go to **Devices** by clicking the icon as shown.

(2) Choose the **heliX®** device you will use for this assay.

(3) Select **Request Control** and wait until the control connection is established. Once this is done, the **Request Control** will turn into **Release Control**.



(4) Select **Eject Trays**.

(5) Remove the chip tray from the compartment and place your biochip in any of the five chip positions (here: position 1). Place a Cleaning Chip (here: position 3), useful for priming the device or a buffer exchange. Push the tray back into the compartment.

(6) In the sample tray, place the 10 mL vial with DI water into position A. Place the ready-to-use 96-well plate in the plate position and make sure that it clicks into place. Push the tray back into the compartment.
Chose **Insert Trays**.

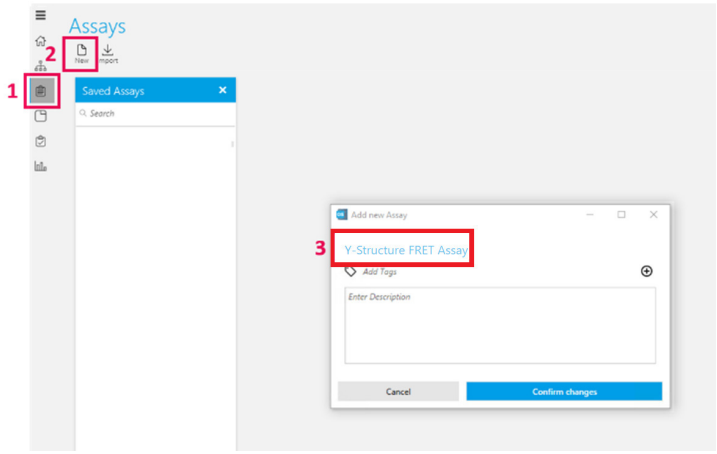
(7) Set the **Temperature** to 15°C and press the arrow to start the temperature control.

Setting up the Y-Structure FRET Kinetics Assay in heliOS

(1) Go to **Assays** by clicking the icon as shown.

(2) Select **New** to create a new assay.

(3) Rename the new assay (here: "Y-Structure FRET Tutorial") and **Confirm Changes**.



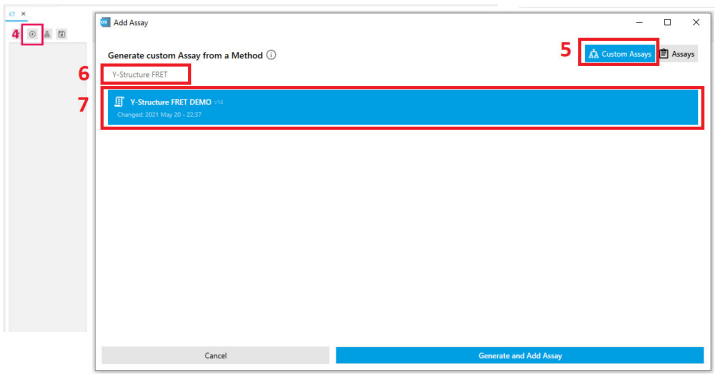
(4) Add a new Assay element by clicking the "**+**" icon.

(5) Go to **Custom methods**.

(6) In the search bar type "Y-Structure FRET Tutorial".

(7) Choose **Y-Structure FRET Tutorial** from the assay list and confirm by clicking.

(8) Click **Add**: The default Y-Structure FRET assay opens automatically.



(9) Select the respective chip position by opening the **Chip** drop-down menu (here: position 1).

(10) The Analysis Type should be "Kinetics.Screening"

(11) Select an available row on the plate (A, B, C)

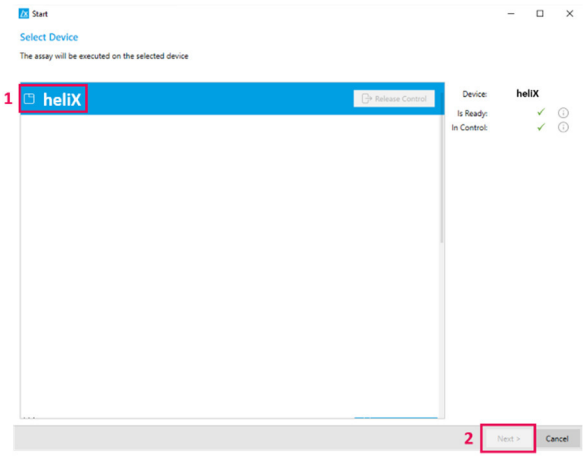
(12) In the **Inflection Point** drop-down menu the default inflection point (IP) of 0.15 V is set. You can change the setting according to the measured IP in your chip status.

(13) **Save** your assay. On the left side of the **Save** icon, click the **Run** button.

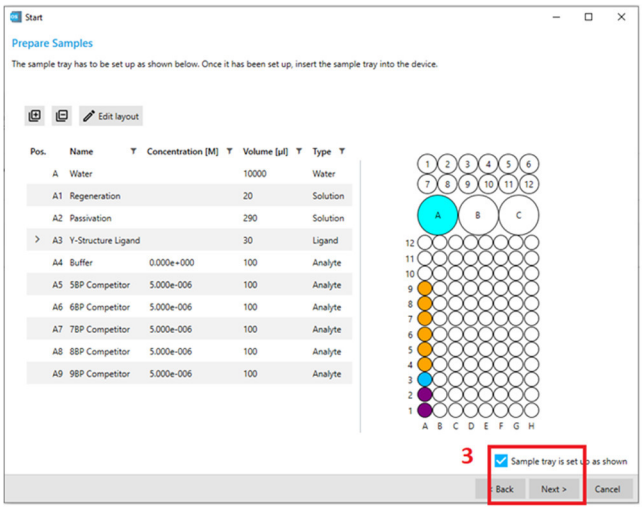
Conducting the Experiment

(1) Select the **heliX®** device which will be used for the measurement.

(2) Press **Next**.

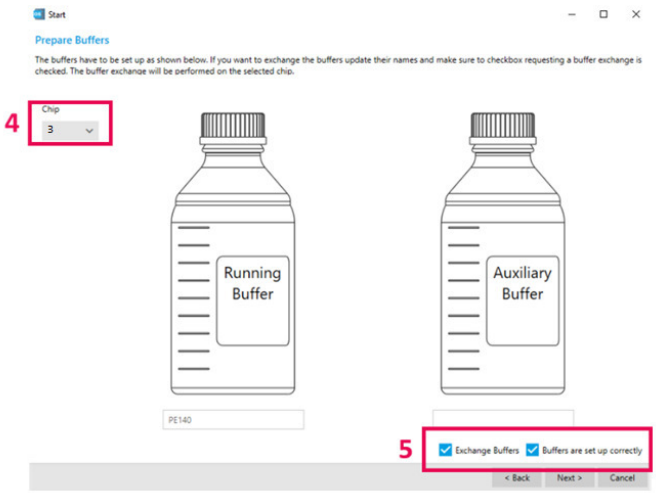


(3) Confirm the sample set-up by ticking the box **Sample tray is setup as shown** and then click **Next**.



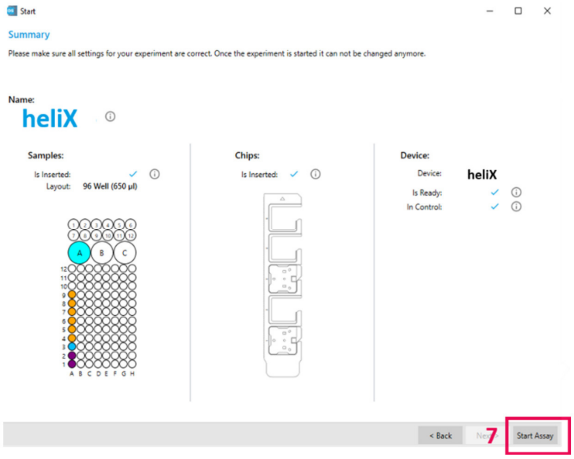
(4) To prime the device with PE140 running buffer, open the **Chip** dropdown menu and select the position where your Cleaning Chip is (here: position 3).

(5) Tick **Exchange Buffers** and **Buffers are set up correctly**. Click **Next**.



(6) In the following window, confirm that the **Chip tray is setup as shown** and click **Next** (screenshot is not shown for this step).

(7) The assay summary shows an overview of the Sample tray, the Chip tray, and the current state of the device. Press **Start Assay**.



Congratulations, you have started your Y-Structure FRET kinetics experiment!

Measurement Steps

Regeneration

The automated regeneration process contains two steps:

1. Denaturation of the double-stranded DNA nanolevers by a basic regeneration solution; this leaves bare single-stranded DNA anchor strands on the electrode surface.

2. Selective hybridization of ***Adapter strands*** onto the surface immobilized anchor strands. ***Adapter 1*** (carrying the Y-Structure ligand) will hybridize only with ***Anchor 1*** (on **Spot 1**) and ***Adapter 2*** (without ligand) only with ***Anchor 2*** (on **Spot 2**). This DNA-encoded addressing ensures selective immobilization of ligand (here: Y-Structure DNA overhang) on **Spot 1** while **Spot 2** is immobilized with the ligand free strand. The injection of prehybridized ***Adapter strands*** can be observed in real-time as a step function in the red and green fluorescence signal as the adapter strands carry a red and green fluorophore.

Binding Kinetics: Association and Dissociation of Analytes

Association and dissociation are performed in static mode and changes in the fluorescence signal are observed in real-time. For a static mode measurement, a fixed potential is applied to keep the DNA nanolevers at an upright position.

The analytes (5, 6, 7, 8 or 9 oligonucleotide competitors) are injected in a series of increasing oligonucleotide length at a fixed concentration of 5 µM. After each analyte injection, a dissociation is performed by flushing running buffer through the microfluidic channel. Association and dissociation times are 12 s and 90 s, respectively. The flow rate is 500 µL/min. Between analyte concentrations, a regeneration of the surface with fresh ligand is not required as the oligonucleotides fully dissociates from the target sequence (Y-Structure overhang) on the surface. Hence, all surface-bound ligands are completely accessible for each analyte injection.

Assay Set-up | heliOS

General workflow of a Y-Structure FRET assay:

1) The ssDNA monolayer of a **switch**SENSE® biochip is hybridized with complementary **Adapter strands** elongated with the Y-Structure format. The Y-Structure contains a complementary ssDNA-overhang ("zipper") as a ligand on each arm. On average, this hybridized zipper sequence leads to a partially closed Y-Structure, which introduces a FRET from the green to the red dye.

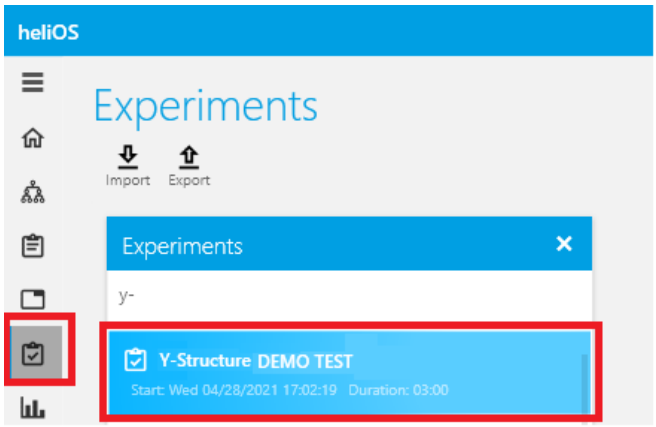
2) Analyte binding measurement of a series of "competitor" oligonucleotides. The competitor sequence is complementary to the zipper of the green arm of the Y-structure and competes with the zipper sequence on the red arm of the Y-Structure. Upon binding of the analyte, the Y-Structure is opened, which abolishes the FRET signal. Reversely, dissociation of the competitor leads to a closing of the Y-Structure and a re-establishing of the FRET.

3) Surface regeneration either by removal of analyte via dissociation (2) during the analyte binding measurement or by hybridization of a fresh DNA Y-Structure (3).

Data Analysis with heliOS

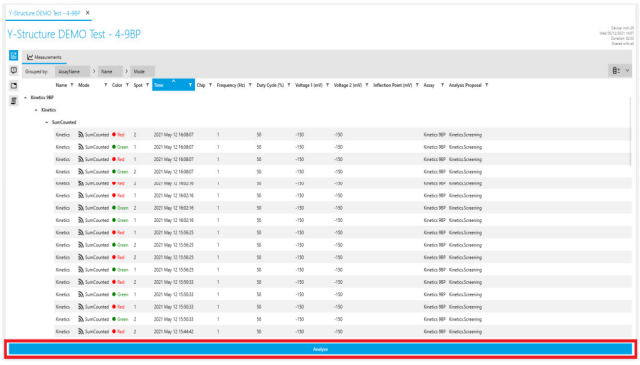
(1) Go to **Experiments** by clicking the icon as shown.

(2) If necessary, download the acquired dataset by clicking the Cloud icon. Once the download is completed, double-click on the dataset.



(3) The dataset is opened automatically. Click on **Analyze**.

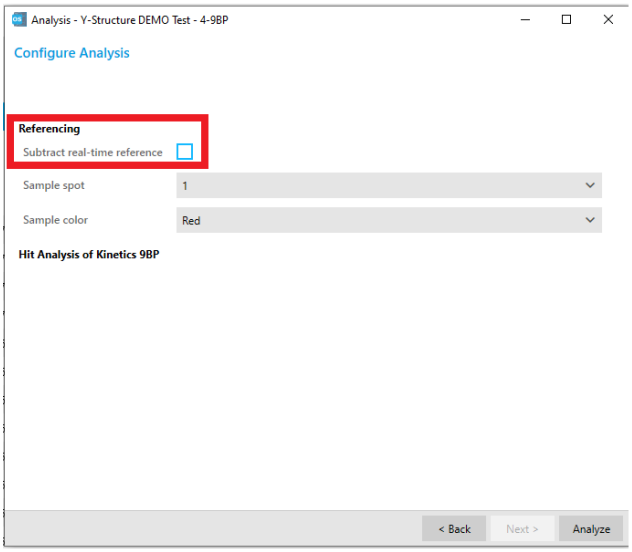
(4) In the pop-up window: Select **Kinetics experiment** and click **Next**. In the next window select **Hit Analysis** and click **Next** (screenshot is not shown for this step).



The next window allows to choose some parameters for the analysis, like the type of referencing.

(5) To perform normalization-based analysis, untick the **Subtract real-time reference** box, keep all other default settings to view the red channel (obtained FRET signal).

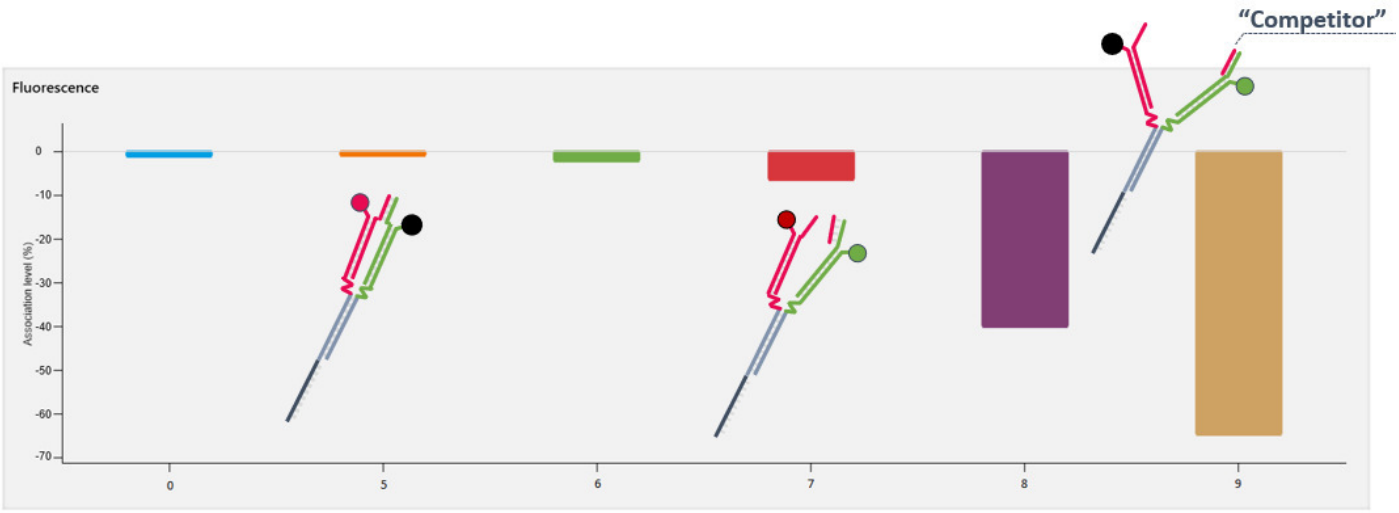
(6) Select **Analyze** to start an automated analysis.



Data Analysis - Additional Information - Hit Screening

A hit-screening analysis (see below) for the five different analytes, the 5-, 6-, 7-, 8- and 9-mer oligos is displayed for the red channel fluorescence change (Acceptor, FRET signal), which shows the loss of red signal in % (Association Level). The red signal represents the transferred FRET signal from the green channel (Donor) to the red (Acceptor) only, as in this assay the red light source is turned off. Hence, signal change in red is only obtained when a change in the Y-Structure formation has occurred. Analyte binding without a FRET change would only be observed in the green channel.

Each trace is normalized (percentage) with respect to its baseline. Hence the buffer or real time referencing are not needed. As the Y-Structure is mainly closed at the beginning of the assay, a reduction in the red fluorescence signal represents an effective competition and opening of the Y-Structure. As shown in Figure 1, a 5- or 6-mer oligo cannot compete with the hybridized 9-mer overhangs on each Y-Structure arm, and the FRET signal remains unmodified. However, with increasing oligo lengths (7- to 9-mer competitor) the FRET signal drops dramatically, as the competition becomes more effective and the Y-Structures are at an open stage.

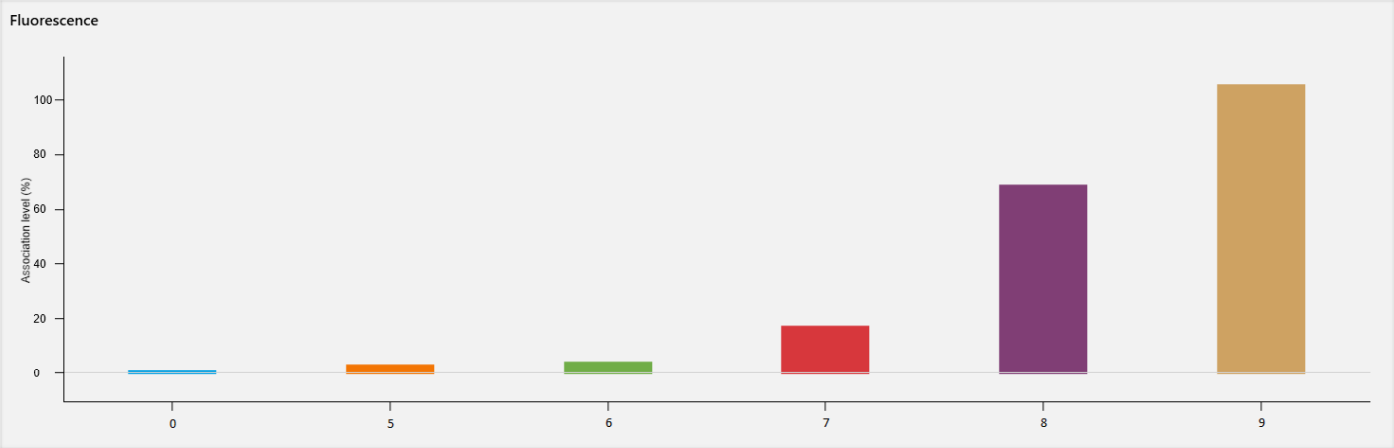


Individual Results

Trace	Name	Association Level (%)	Dissociation Level (%)	Well
	Buffer	-1.14	-2.21	A4
	5BP Competitor	-919 E-3	-2.57	A5
	6BP Competitor	-2.16	-2.23	A6
	7BP Competitor	-6.58	-2.13	A7
	8BP Competitor	-40.0	-2.28	A8
	9BP Competitor	-64.6	-1.82	A9

Figure 1 | **heliOS** hit screening data analysis of the red fluorescence signal (FRET Signal). The 5bp Competitor cannot open the Y-Structure, while starting from the 6BP Competitor, the Y-Structure is forced to open the arms as the competitors' length increases. This distance increase between the Y-Structure arms leads to a decrease of the FRET signal as the energy transfer from the green dye (donor) to the red dye (acceptor) is attenuated. This distance increase is due to the increased affinity of the longer oligos (analyte) towards the hybridized 9-mer overhang present on the green Y-Structure arm and an increased competition with the red 9-mer overhang arm.

The same analysis can be performed for the green channel (donor) and shows the rehabilitation of the green donor emission signal in absence of the red acceptor during the opening of the Y-Structure (a flipped version of the upper plot).



Individual Results

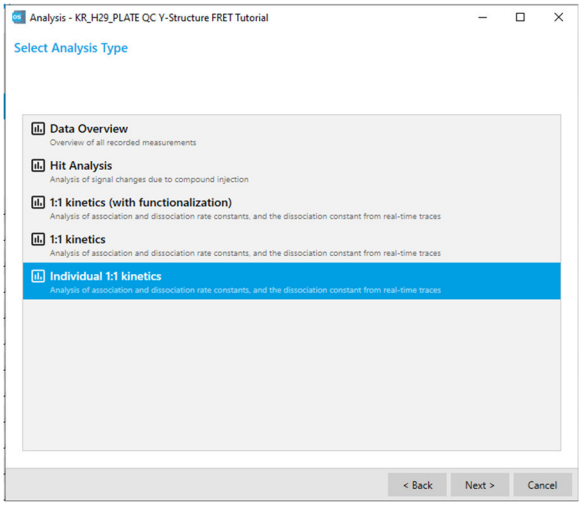
Trace	Name	Association Level (%)	Dissociation Level (%)	Well
	Buffer	614 E-3	1.44	A4
	5BP Competitor	2.81	1.75	A5
	6BP Competitor	4.03	1.72	A6
	7BP Competitor	17.1	1.60	A7
	8BP Competitor	68.8	1.50	A8
	9BP Competitor	105	1.72	A9

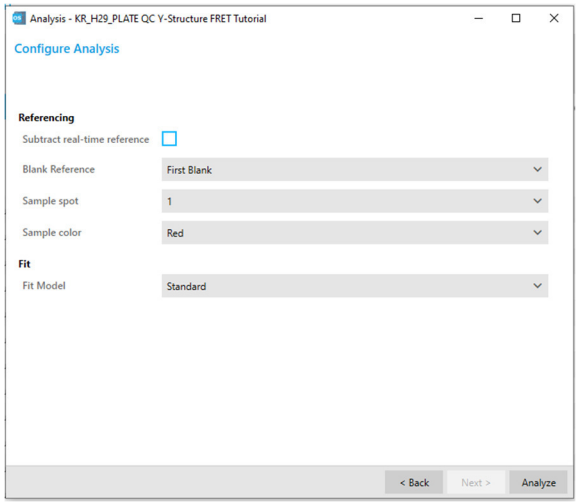
Figure 2 | **heliOS** hit screening data analysis of the green fluorescence signal (donor signal) showing the recovery of the donor signal when FRET is abrogated due to the opening of the Y-Structure.

Data Analysis - Additional Information – 1:1 Individual Kinetics

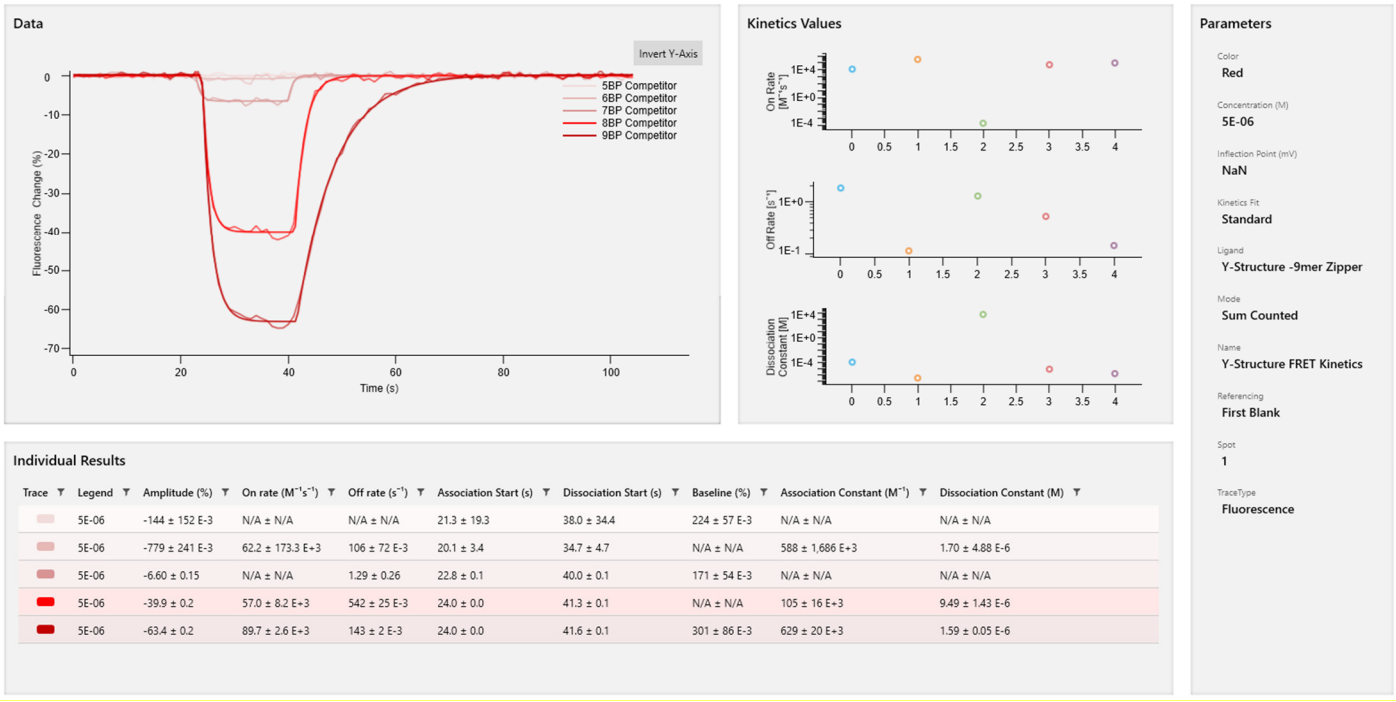
Considering that this assay is performed using one blank run and 5 different analytes (5-, 6-, 7-, 8- and 9-mer oligos) only an individual 1.1 kinetics fit can be performed (either for the red or green signal) to obtain binding kinetics data for each competitor, separately.

This evaluation can be carried out as following: click on the **Analyze** tab (as done previously for the hit-screening analysis), select “Individual 1:1 Kinetics”, as shown in the left screenshot. Untick the “subtract real time reference” box and select the preferred signal (red or green), as shown in the screenshot on the right.



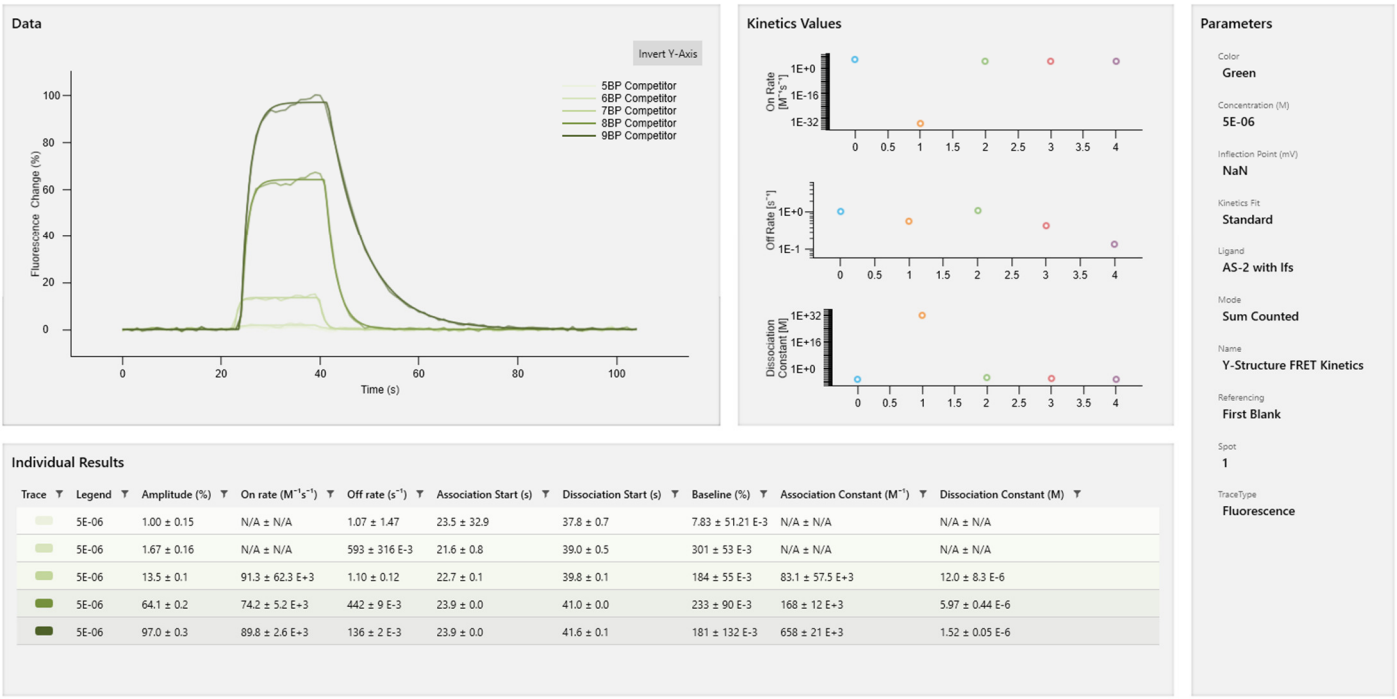


The result is a series of blank-referenced individual kinetic fits, one trace for each oligo, as shown in Figure 3 (Red channel) and Figure 4 (Green channel).



Trace	Legend	Amplitude (%)	On rate ($M^{-1}s^{-1}$)	Off rate (s^{-1})	Association Start (s)	Dissociation Start (s)	Baseline (%)	Association Constant (M^{-1})	Dissociation Constant (M)
	5E-06	-144 ± 152 E-3	N/A ± N/A	N/A ± N/A	21.3 ± 19.3	38.0 ± 34.4	224 ± 57 E-3	N/A ± N/A	N/A ± N/A
	5E-06	-779 ± 241 E-3	62.2 ± 173.3 E+3	106 ± 72 E-3	20.1 ± 3.4	34.7 ± 4.7	N/A ± N/A	588 ± 1,686 E+3	1.70 ± 4.88 E-6
	5E-06	-6.60 ± 0.15	N/A ± N/A	1.29 ± 0.26	22.8 ± 0.1	40.0 ± 0.1	171 ± 54 E-3	N/A ± N/A	N/A ± N/A
	5E-06	-39.9 ± 0.2	57.0 ± 8.2 E+3	542 ± 25 E-3	24.0 ± 0.0	41.3 ± 0.1	N/A ± N/A	105 ± 16 E+3	9.49 ± 1.43 E-6
	5E-06	-63.4 ± 0.2	89.7 ± 2.6 E+3	143 ± 2 E-3	24.0 ± 0.0	41.6 ± 0.1	301 ± 86 E-3	629 ± 20 E+3	1.59 ± 0.05 E-6

Figure 3 | **heliOS** 1:1 individual kinetics data analysis of the red (acceptor) fluorescence signal. The 5 analytes (5-, 6-, 7-, 8- and 9-mer oligos) are normalized and blank referenced. The kinetic curves of 8- and 9-mer oligos can be fitted and used to obtain binding kinetics data.



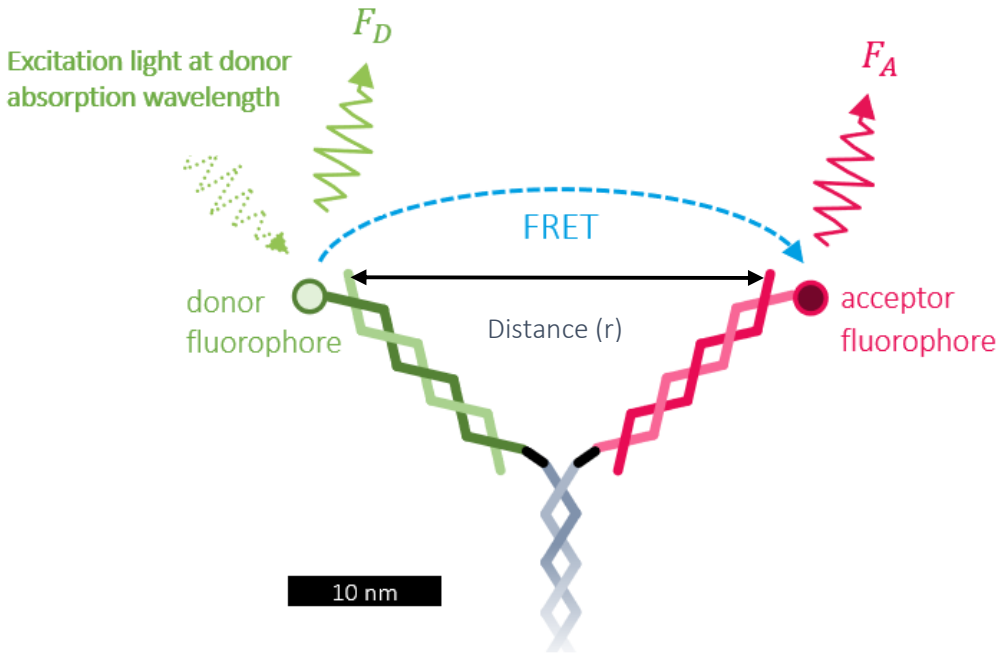
Individual Results

Trace	Legend	Amplitude (%)	On rate ($M^{-1}s^{-1}$)	Off rate (s^{-1})	Association Start (s)	Dissociation Start (s)	Baseline (%)	Association Constant (M^{-1})	Dissociation Constant (M)
	5E-06	1.00 ± 0.15	N/A ± N/A	1.07 ± 1.47	23.5 ± 32.9	37.8 ± 0.7	7.83 ± 51.21 E-3	N/A ± N/A	N/A ± N/A
	5E-06	1.67 ± 0.16	N/A ± N/A	593 ± 316 E-3	21.6 ± 0.8	39.0 ± 0.5	301 ± 53 E-3	N/A ± N/A	N/A ± N/A
	5E-06	13.5 ± 0.1	91.3 ± 62.3 E+3	1.10 ± 0.12	22.7 ± 0.1	39.8 ± 0.1	184 ± 55 E-3	83.1 ± 57.5 E+3	12.0 ± 8.3 E-6
	5E-06	64.1 ± 0.2	74.2 ± 5.2 E+3	442 ± 9 E-3	23.9 ± 0.0	41.0 ± 0.0	233 ± 90 E-3	168 ± 12 E+3	5.97 ± 0.44 E-6
	5E-06	97.0 ± 0.3	89.8 ± 2.6 E+3	136 ± 2 E-3	23.9 ± 0.0	41.6 ± 0.1	181 ± 132 E-3	658 ± 21 E+3	1.52 ± 0.05 E-6

Figure 4 | **heliOS** 1:1 individual kinetics data analysis of the green (donor) fluorescence signal. The 5 analytes (5-, 6-, 7-, 8- and 9-mer oligos) are normalized and blank referenced. The kinetic curves of 8- and 9-mer oligos can be fitted and used to obtain binding kinetics data.

Additional Information

Förster resonance energy transfer (FRET) is a distant dependent energy transfer mechanism between two fluorophores, from a donor fluorophore (here: green) to an acceptor fluorophore (here: red). Energy transfer occurs when the donor dye is excited (here: LED with respective filter set) while in close proximity to an acceptor dye that is sensitive to the emitted energy (wavelength) of the donor (see below).



The FRET efficiency (E) is hereby inversely proportional to the sixth power of the distance (r) between donor and acceptor, with R_0 being the Förster distance between the pair of donor and acceptor, which makes this energy transfer a highly sensitive tool to detect distant-dependent changes.

$$E = \frac{1}{1 + (r/R_0)^6}$$

In this kit, FRET is used to probe the distance between the green and the red arm to determine if the Y-Structure is open (ligands apart) or closed (ligands bound to each other). This allows characterization of compounds that either inhibit (e.g., inhibitors of protein-protein dimerization) or facilitate this process. Quantifying the amount of FRET by measuring the change of the red (acceptor) fluorescence allows to validate the efficiency of inhibitors or analytes that facilitate FRET.

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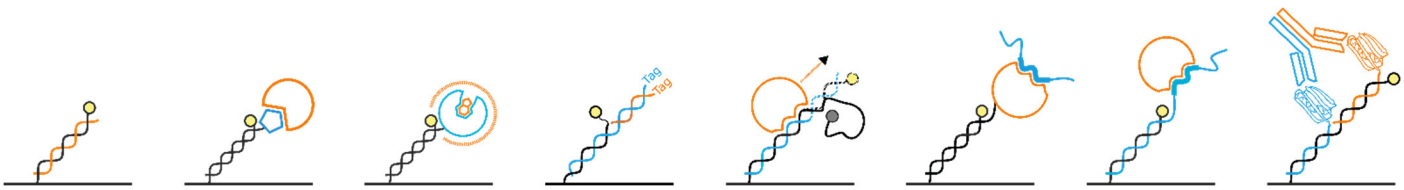

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Instruments and biochips are engineered and manufactured in Germany.