

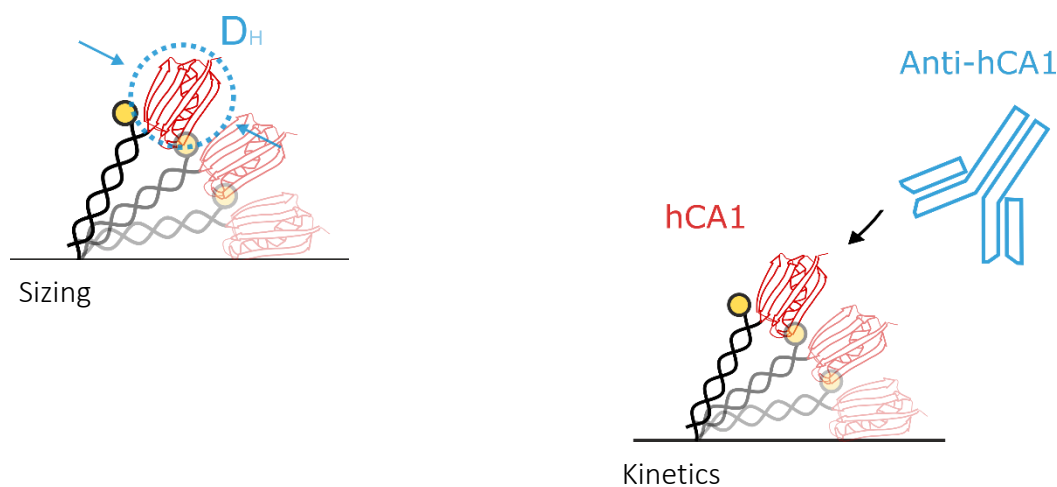
switchSENSE® Demo Kit

DK-Anti-hCA: Protein - Protein (D_H | k_{ON} | k_{OFF})

Sizing of Carbonic Anhydrase, Association and Dissociation of an Antibody

Aims

- Familiarization with the software tools for performing switchSENSE® experiments
- Determination of the hydrodynamic diameter (D_H) of human Carbonic Anhydrase 1 (hCA1)
- Determination of the association and dissociation rate of a hCA1-specific antibody (k_{on} , k_{off} , K_D)



Workflow

Design your experiment



switchBUILD

Step-by-step guidance through the workflow

Perform your experiment



switchCONTROL

Finetuning of the experiment (if needed)

Analyze your results



switchANALYSIS

Simple drag'n'drop format for efficient data interpretation

Product description

Product Code DK-AP-1-B48

Measurement Time ~2 h 32 min

In this experiment, the binding of an antibody to human Carbonic Anhydrase 1 (hCA1) is analyzed. We start with immobilizing hCA1 on the biosensor surface. During stopped flow we can size the protein. Next, we observe the association of three consecutive concentrations of the antibody to hCA1. Finally, dissociation of the antibody is recorded after association of the highest concentration.

Before getting started, transfer one aliquot of each sample listed in Table 1 to the fridge to ensure slow thawing.

TABLE 1 | Contents and storage information



Material	Cap	Amount	Storage	Comments
cNL-A48 / cNL-B48 (500 nM each)	blue	3 x 65 µL	-20°C	in PE40
cNL-A48 / cNL-B48-hCA1 (200 nM each)	green	3 x 90 µL	-20°C	in PE40
Anti-hCA1 (10 nM)	red	3 x 310 µL	-20°C	in PE40

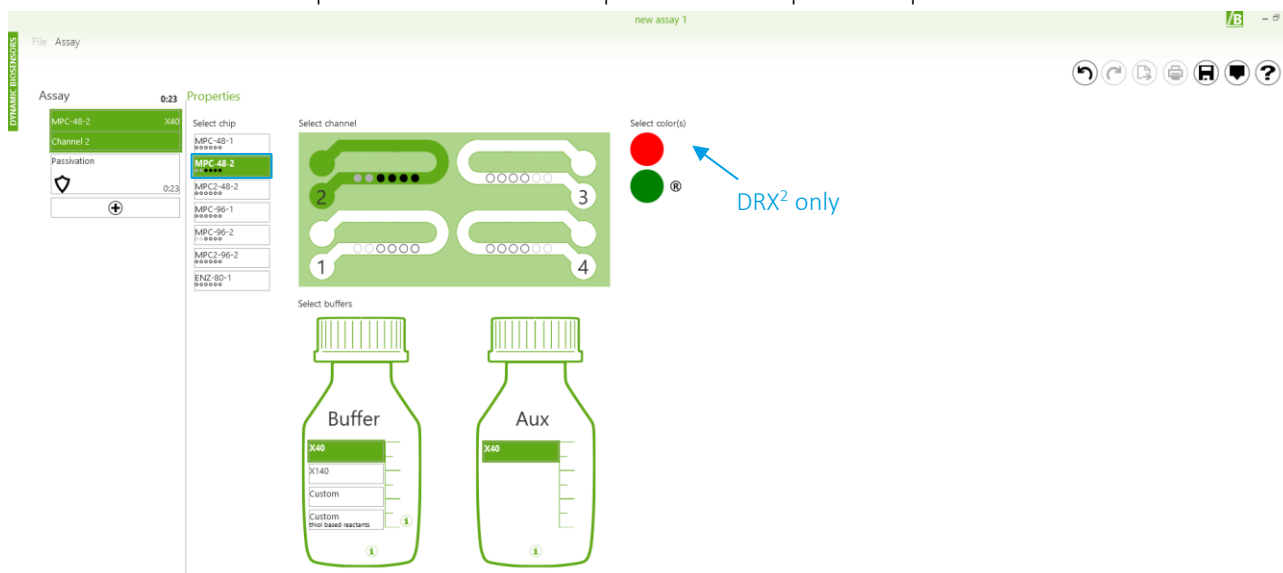
TABLE 2 | Additional material required


Material	Cap	Amount	Storage	Comments
MPC-48-2 biochip	-	1	2-8°C	use in DRX or DRX ²
PE40 buffer (1x): 10 mM NaH ₂ PO ₄ /Na ₂ HPO ₄ , pH 7.4, 40 mM NaCl, 50 µM EDTA, 50 µM EGTA, 0.05 % Tween 20, sterile filtered	transparent	2 x 50 mL	2-8°C	Running buffer Aux buffer
DI Water	transparent	1 x 12 mL	2-8°C	
Passivation solution (10x)	yellow	1 x 1 mL	2-8°C	dilute in 9 ml DI water
1x Regeneration solution	transparent	1 x 12 mL	2-8°C	

Large glass vials (10 mL), small glass vials (1.5 mL), caps required. For *in vitro* use only.

Planning the experiment

1. Start switchBUILD by clicking on the  icon on the desktop and click on **+ New** to create a new assay. The interface is arranged in two different columns: *Assay* and *Properties*. The *Assay* column shows all elements of your assay. By default, it includes a panel to choose the basic settings of your assay and a *Passivation* element, which should be run before the measurement to prevent unspecific binding to the biosensor surface. Next to *Assay* the estimated runtime of your assay is shown. In the *Properties* column, the individual measurement parameters for each assay element can be set. The info button  always provides further information about the current topic.
2. Now, let's start with defining the general *Properties* of your assay by selecting the first panel of your assay. Choose the **flow channel** you want to run the experiment in by clicking on the channel – the selected channel is highlighted in green. Furthermore, the running buffer *Buffer* and auxiliary buffer *Aux* can be specified. For this demo the default settings are appropriate. To specify the type of Biochip used, select "MPC-48-2" from the *Select chip* list, which indicates that it is a Multi Purpose Chip (MPC) with a functionalized DNA-length of 48 base pairs and two different DNA sequences. The two sequences are spatially separated with sequence A on electrodes 1 and 2, and sequence B on electrodes 3-6. You can use within one measurement sequence A as control and sequence B for the specific sample of interest.



3. To add assay elements to the sequence, click  in the *Assay* column and choose the desired element. As one of the goals of this experiment is to determine binding kinetics, we need a **Kinetics** element.



- In the next step, you should define the *Properties* of the Kinetics assay. In the upper section general information about your interaction experiment is requested. Select **Conjugate Hybridization** for *Immobilization*, which assumes that your ligand is conjugated to the complementary DNA (cNL-B48-hCA1). Keep **Dynamic Mode** as *Measurement* type. In this mode, the DNA is switched during the measurement. Update the name of your immobilized *Ligand* to **hCA1** and change its concentration to **200 nM**, the molecular weight is about **29 kDa**. The name of your *Analyte* is **Anti-hCA1**; since it is an antibody it has a molecular weight of about **150 kDa**.

- In the *Interaction* tab you are asked to give an estimation of the expected interaction parameters (k_{on} , k_{off} , K_D). You can either choose saved settings via **PRESETS** or enter custom values. Please enter the following parameters, either by moving the sliders or by typing the values in the corresponding fields: $K_D = 5.0E-10$ M, $k_{on} = 6.0E+06$ M⁻¹s⁻¹, $k_{off} = 3.0E-03$ s⁻¹. When using the slider, one of the three values will always be fixed, as each value is dependent on the other two: $K_D = \frac{k_{off}}{k_{on}}$. The values provided are used to predict the Fraction Bound [%] shown in the graph on the right, which is updated on the fly when you make changes in the next step:
- As experimental parameters we keep **3 concentrations** of anti-hCA1, *starting from* the stock concentration of **1E-8 M**, which will be diluted with a *factor* of **2**. The resulting concentrations are displayed below the graph on the right. Next enter the *Association volume* of **110 µL**, with a pump rate of **100 µL/min**. The time is the result of volume divided by pump rate and can be used for setting the volume as well. The *Dissociation* should be run with **4000 µL** at a pump rate of **200 µL/min** with the syringe pump (SP).

- In the third section, you are asked to select the measurement spots that will be used in your experiment. The selected spots are colored in green. For this demo, choose **one measurement spot** out of spots 3-6 and select it for both **Association and Dissociation**. From **Stopped Flow** measurement data you can do a size analysis of your conjugate and analyte, for this purpose activate at least one spot out of spots 3 - 6 and check the **perform as Sizing** box. To control for unspecific binding one of the measurement spots 1 or 2 could be selected as well.

In addition, you can decide if the flow channel should be functionalized with fresh ligand after each concentration. This includes a regeneration, in which the biochip is first washed with a proprietary regeneration solution (SOL-REG-12-1) to ensure a fully single-stranded state of all covalently attached DNAs. Subsequently, fresh conjugate DNA is re-hybridized. It is advisable to perform regenerations between concentrations if no complete dissociation of the analyte has taken place

beforehand. Since in this demo we are running three concentrations of strongly binding antibody, please check the checkbox **with Regenerations**. Finally, you can save assay run time by checking **only last concentration**, which deactivates all dissociations but after the last (highest) concentration of analyte.

Measurement Spots

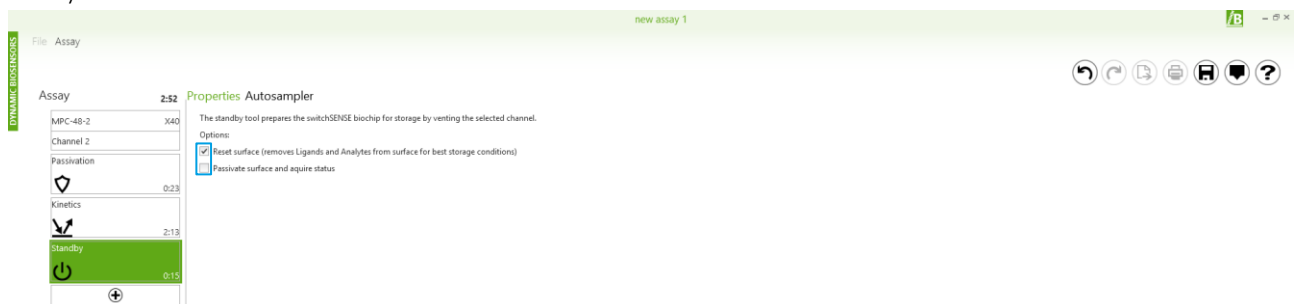
Association	1	2	3	4	5	6
Stopped Flow	1	2	3	4	5	6
Dissociation	1	2	3	4	5	6

with Regenerations ⓘ

perform as Sizing ⓘ

only last concentration ⓘ

- At last, add a **Standby** assay element to your Assay sequence and only choose **Reset surface**. This step removes the ligand, regenerates the chip with bare DNA, fills the measurement channel with air and thereby prepares the biochip for storage after your measurement.



- Save your newly designed assay as a DBB file by clicking on and name the file "DK-Anti-hCA1".
- You will be taken to the *Autosampler* tab, where you can see how the autosampler of the DRX should be loaded for the experiment. In the middle column you can find a list of all solutions and their concentrations needed for your measurements. When you click on an item in the solution list, its respective position in the autosampler display (right column) is indicated with a green circle.


Sample	Volume (µl)	Conc (M)	Type	Vial
A1 Waste	-760		Waste	Large
B1 Water	filled		Water	Large
C1 Passivation solution	2150		Solution	Large
A2 X40	1500		Solution	Large
A5 Regeneration solution	140		Solution	Small
B5 cNL-A40 and B40	65	5.00E-07	Ligand	Small
C5 hCA1	90	2.00E-07	Ligand	Small
A10 Anti-hCA1	150	2.50E-09	Analyte	Small
B10 Anti-hCA1	150	5.00E-09	Analyte	Small
C10 Anti-hCA1	150	1.00E-08	Analyte	Small

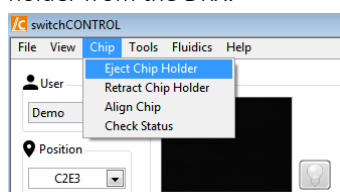
The autosampler display grid (rows 1-10, columns A-F) shows the following positions highlighted with colored circles:

- Row 1: A (black), B (blue), C (yellow)
- Row 2: A (purple)
- Row 5: A (green), B (blue), C (blue)
- Row 6: A (orange), B (orange), C (orange)

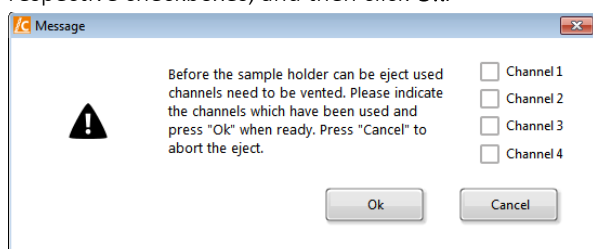
Congratulations, you just designed your own **switchSENSE®** experiment!

Conducting the experiment

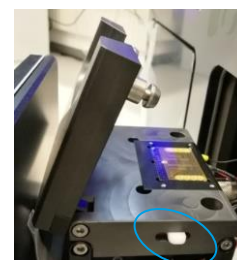
1. Start **switchCONTROL** by clicking on the  icon on the desktop and enter **your name** when prompted (the measured data will be automatically saved in a folder with your name).
2. To insert a new chip, click on **Chip** → **Eject Chip Holder** in the upper left corner of the software window to eject the chip holder from the DRX.



3. The following message will pop up. Indicate which channels have been used on the inserted biochip by checking their respective checkboxes, and then click **Ok**.





4. After venting, the chip holder will be ejected. Open the chip holder by pushing both white sliders on the right to the back. Use the vacuum pen to remove the chip currently in place and insert your **MPC-48-2** chip. Make sure to place it with the label facing upwards. Once the chip is in place, close the chip holder while pushing the white slider back.

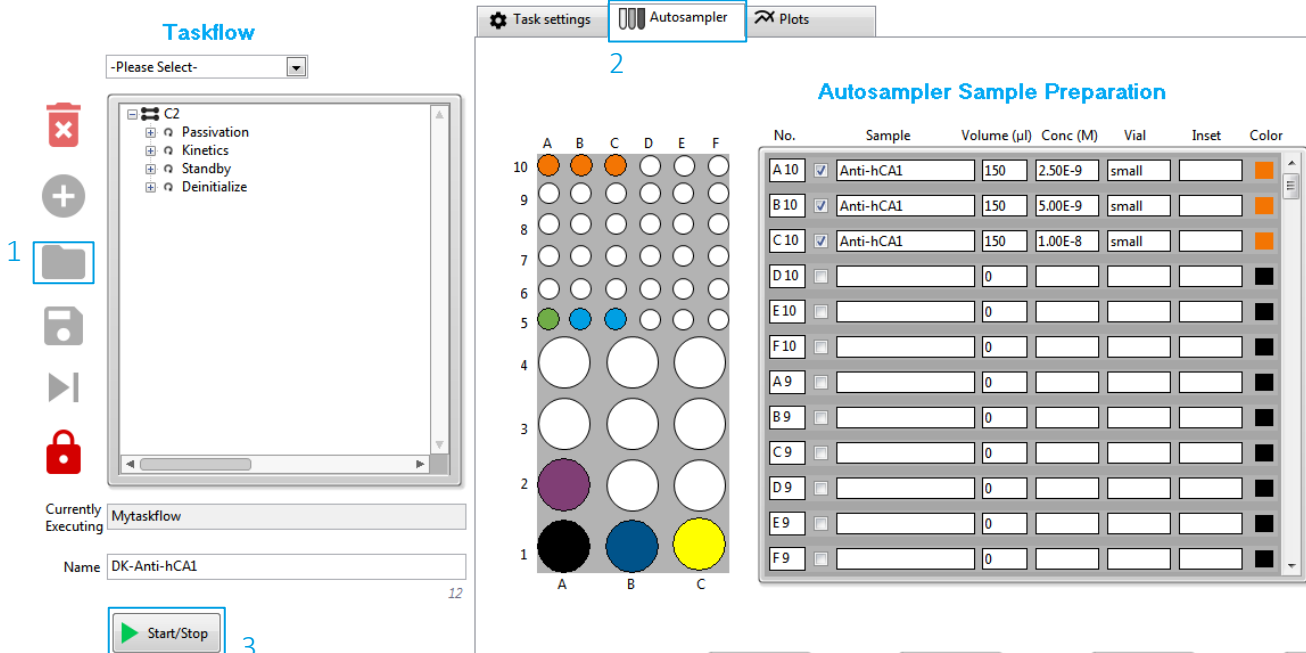


5. Click on **Chip** → **Retract Chip Holder** to retract the new biochip into the DRX and follow the instructions of the **Retract assistant**. Enter the Chip-ID as shown on the packaging of your biochip. A message will pop up asking if the chip should be **aligned** – the alignment is necessary to precisely position the electrodes for measurement. An alignment should be performed every time a chip is placed into the DRX (except for a Cleaning chip which is not used for measurements). The next message will ask you if you wish to **prime** the microfluidics system with the running buffer – the priming should be done if this is your first measurement of the day or if your running buffer is different from that of the prior measurement. Make sure that there are two bottles of PE40 buffer connected to the **Buffer** and **AUX** reservoirs on the top left (exterior) of the instrument. Finally, you are asked if you want to check the status of the electrodes and regenerate the channel before starting the experiment. These steps are not necessary for the purposes of this demo and the default settings can be maintained. The alignment and priming will take a few minutes. You can follow the alignment by watching the small camera window on the top left of the software window, where you will see the focusing on the four alignment crosses.



6. While the alignment and priming are taking place, please prepare the samples as indicated in switchBUILD. **Prepare the anti-hCA1 antibody dilutions** by
 1. mixing 150 μ l of the stock solution with 150 μ l of PE40 buffer (= 5 nM).
 2. Repeat by mixing 150 μ l from 1. with 150 μ l of PE40 buffer (= 2.5 nM).


7. **Load your assay** *DK-Anti-hCA1* into switchCONTROL by clicking on  at the left of the software window {1}. Next, click on the **Autosampler** tab {2} to view the sample positions listed within the script, and load the sample solutions into the autosampler accordingly. Close the autosampler door and start the measurement by clicking on  {3}.





The screenshot displays the software interface for loading and starting an assay. On the left, the **Taskflow** window shows a list of tasks: C2, Passivation, Kinetics, Standby, and Deinitialize. A folder icon is highlighted with a blue box and labeled '1'. Below the taskflow, the 'Name' field contains 'DK-Anti-hCA1' and a 'Start/Stop' button is highlighted with a blue box and labeled '3'. The main window has the **Autosampler** tab selected, indicated by a blue box and labeled '2'. This tab shows a 10x6 grid representing sample positions (A-F, 1-10) with colored circles indicating the loaded samples. To the right, the **Autosampler Sample Preparation** table is shown, detailing the sample information for each position.

No.	Sample	Volume (μ l)	Conc (M)	Vial	Inset	Color
A 10	<input checked="" type="checkbox"/> Anti-hCA1	150	2.50E-9	small		Orange
B 10	<input checked="" type="checkbox"/> Anti-hCA1	150	5.00E-9	small		Orange
C 10	<input checked="" type="checkbox"/> Anti-hCA1	150	1.00E-8	small		Orange
D 10	<input type="checkbox"/>	0				Black
E 10	<input type="checkbox"/>	0				Black
F 10	<input type="checkbox"/>	0				Black
A 9	<input type="checkbox"/>	0				Black
B 9	<input type="checkbox"/>	0				Black
C 9	<input type="checkbox"/>	0				Black
D 9	<input type="checkbox"/>	0				Black
E 9	<input type="checkbox"/>	0				Black
F 9	<input type="checkbox"/>	0				Black

Measurement Steps

If working with a DRX² DualColor instrument, choose to display the **red dye only** by clicking on the color block next to *Display* above the measurement plots .

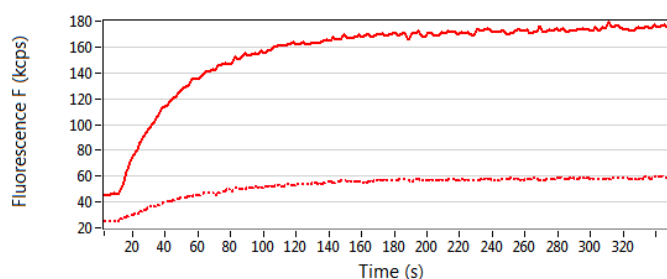
- Passivation and Status

Before starting an experiment, the biosensor surface needs to be passivated to prevent unspecific surface binding, and thus enable maximal DNA-switchability. During the passivation step, the chip is incubated in passivation solution and the DNA levers are switched with a frequency of 0.2 kHz. A positive potential leads to a low fluorescence signal (as the fluorophore lies close to the surface), while a negative potential leads to a high fluorescence signal (as the fluorophore is positioned away from the surface). The *passivation* step takes 10 minutes and should be run completely if the channel had not already been passivated on the same day. If the channel has already been passivated, you can skip this step after a few minutes by clicking on . To do so, the taskflow needs to be unlocked first by clicking on .

After the passivation the chip will be subjected to a *status* diagnosis: Each electrode is calibrated (see below) and the DR and Fluorescence values are recorded. Use this information in case electrodes need to be changed.

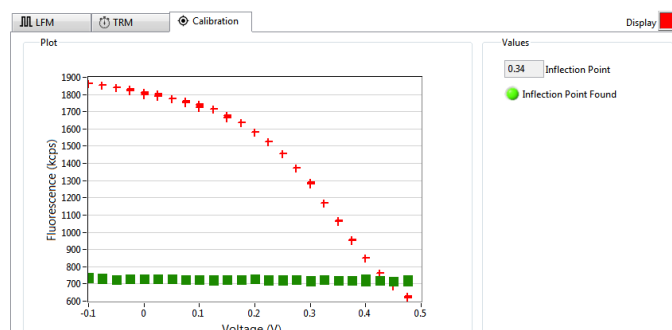
- Functionalization

You will be able to follow the functionalization of the chip with first the reference complement DNA and later the hCA1-conjugated complement DNA in real-time in the **red channel**. Before the *functionalization*, the dsDNA is denatured by the injection of a *regeneration* solution (high pH), therefore the measurement starts with the switching of ssDNA. After a short while, the fresh complementary strand is injected into the flow channel and the ssDNA is hybridized to form dsDNA. As dsDNA is more rigid than ssDNA, the fluorophore is pushed further away from the surface during switching. Consequently, the switching amplitude increases during the hybridization. The functionalization takes about 15 minutes for completion.

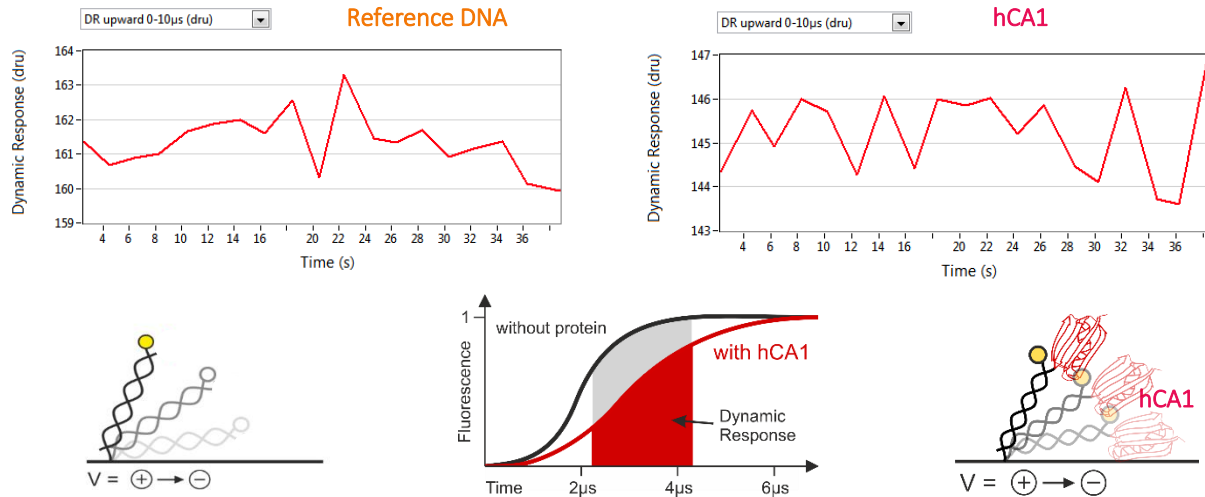


- Calibration and Dynamic Response in Stopped Flow

After hybridization of naked complement DNA, and later of hCA1-conjugated DNA, a *calibration* measurement determines the inflection point of the DNA on the selected electrode. This calibration measurement records the change of fluorescence intensity in response to the applied voltage. More positive voltages attract the negatively charged DNA and orient the DNA nanolevers closer to the quenching gold surface.



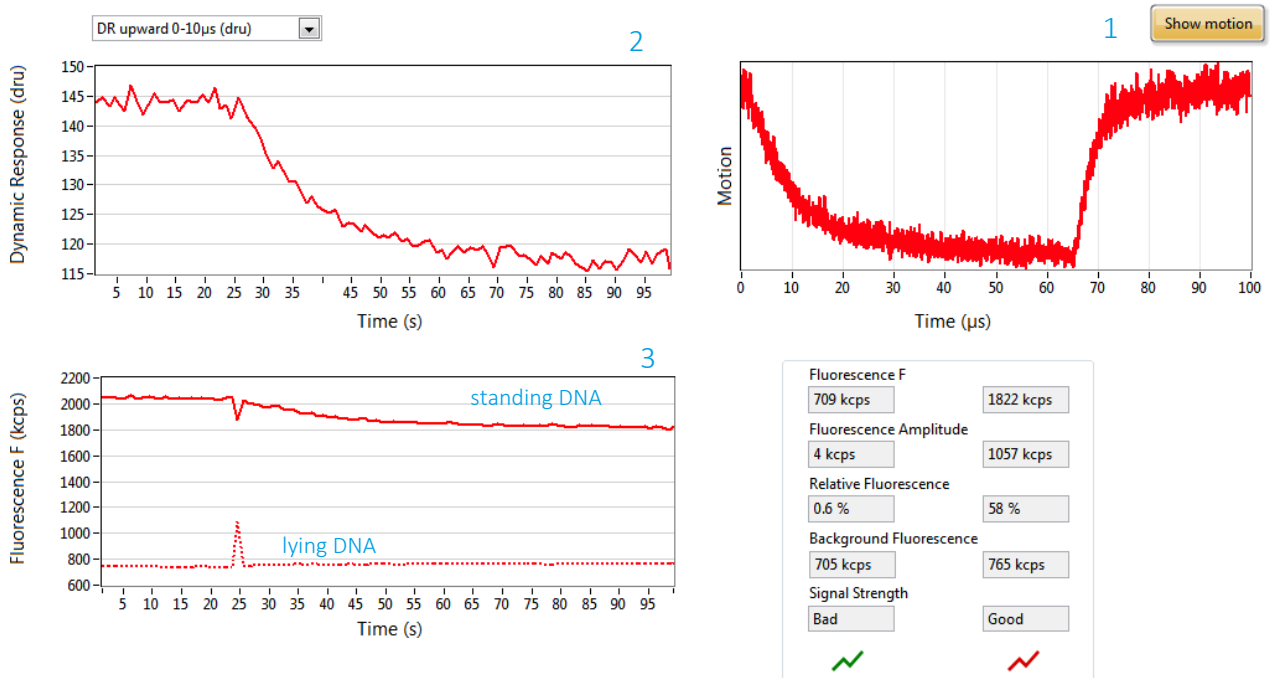
Subsequently, the Dynamic Response is measured during *Stopped Flow* with voltages according to the previously determined inflection point. The Dynamic Response (DR upwards 0 - 10 μ s), plotted top left, is a measure of the switching speed of the nanolever and is derived from its time-resolved upward motion. The switching speed should be reduced on electrode spots 3 – 6 after the functionalization with hCA1, compared to naked complementary DNA, as the bound protein increases the frictional drag of the DNA nanolever during switching.



- Association and Dissociation of Anti-hCA1

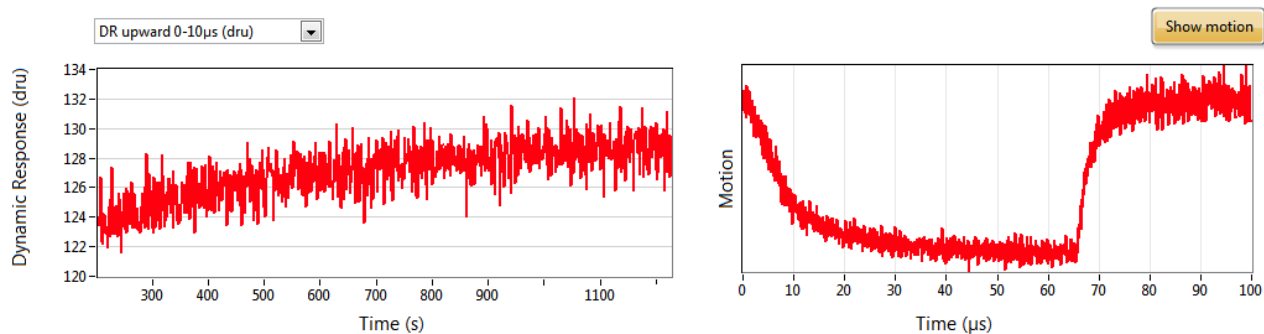
To measure the binding kinetics, the DNA nanolevers are actuated at a high frequency (10 kHz). When clicking on the **Show motion** button, you will be able to see the fluorescence signal corresponding to the time resolved upward and downward motion of the DNA strands {1}. Additionally to the *Dynamic Response* {2}, the maximum (F_{up}) and minimum (F_{down}) *Fluorescence* intensities are plotted below {3}, representing the highest position the DNA can reach when standing up and the lowest when lying down, respectively.

For 30 secs, the DR of the nanolevers functionalized with hCA1 will be measured. After that, the anti-hCA1 solution is injected. Now you can follow the binding of anti-hCA1 to the hCA1-functionalized DNA in real-time: As the binding of the antibody slows down the motion of the nanolever (due to the increased hydrodynamic friction of the bound complex in reference to the naked functionalized DNA), the DR decreases until equilibrium is reached.






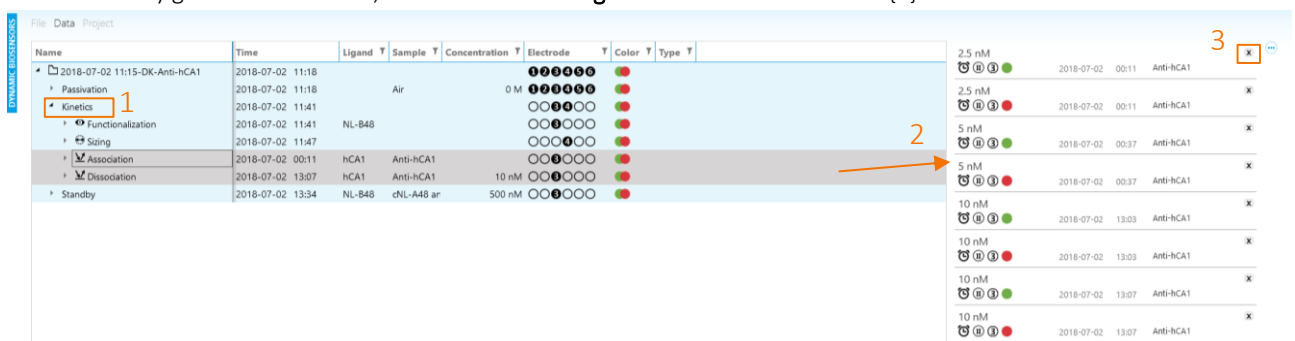
To determine the size of the anti-hCA1, a short Stopped flow measurement is conducted after its association has been completed. The Stopped flow measurement of anti-hCA1 is performed in the same manner as that of the hCA1-functionalized DNA nanolevers and measures the change in DR due to increased friction (see above).

After the association of the last (10 nM) antibody concentration is completed, running buffer is injected into the microfluidic channel. You can follow the *dissociation* of the antibody in real-time: As the unbinding of the antibody leads to an increased switching speed, the Dynamic Response increases. Since anti-hCA1 is a strong binder, this dissociation process is slow.



Data analysis with the switchANALYSIS software

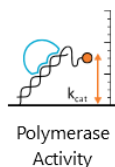
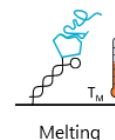
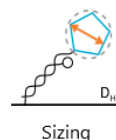
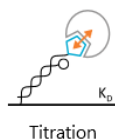
1. Start switchANALYSIS by clicking on the  icon on the desktop and click on  to create a new assay. Now you can load your data by clicking on **IMPORT EXPERIMENT FILE** in the lower left corner and selecting your zipped measurement data, which is saved in > folder with your username > subfolder with the date.
2. You will find the assay structure of your switchBUILD script (Passivation – Kinetics – Standby) when expanding the measurement file (indicated by the start time of your measurement) by **clicking on the triangles**. Further expansion of the *Kinetics* dataset {1} reveals data for the Functionalization, Sizing (from the Stopped Flow measurements), and the Association and the Dissociation of anti-hCA1 antibody. Drag-and-drop the folders **Association** and **Dissociation** into the right column to select the data {2}. Both the red and green channel recorded data during the measurements, however, we did not use any green-labeled DNA, therefore **delete all green** marked data with  {3}.




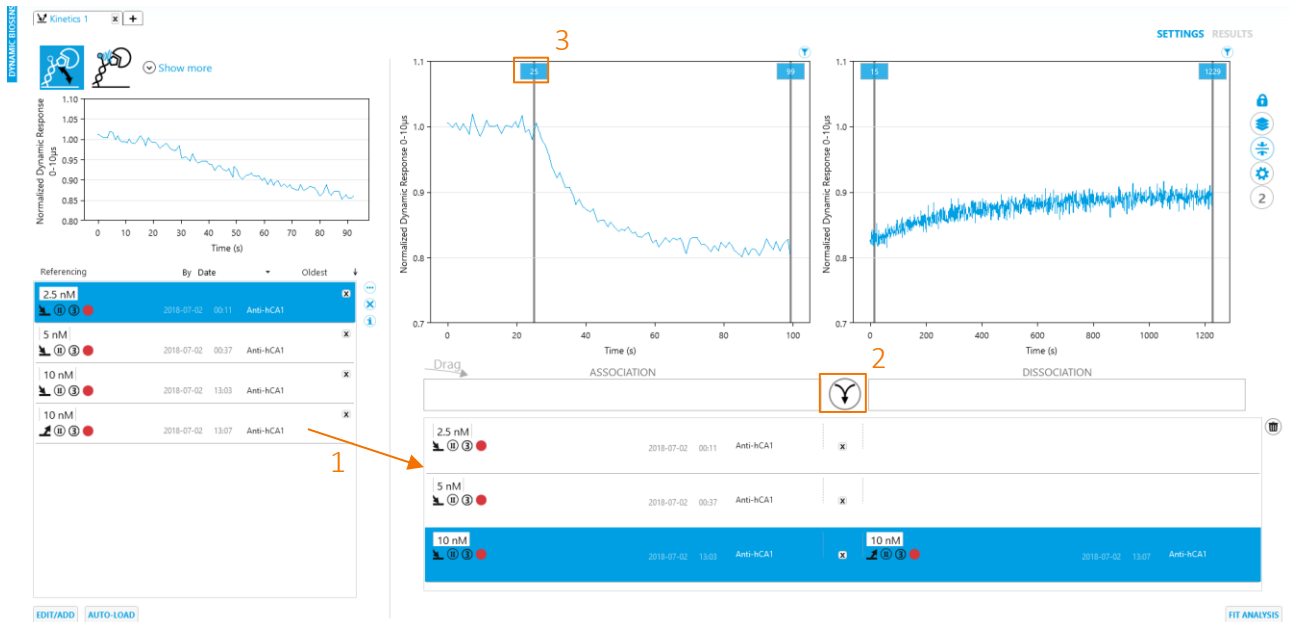
Name	Time	Ligand	Sample	Concentration	Electrode	Color	Type
2018-07-02 11:15-DK-Anti-hCA1	2018-07-02 11:18				●●●●●●●●	●●	
Passivation	2018-07-02 11:18		Air	0 M	●●●●●●●●	●●	
Kinetics	2018-07-02 11:41				○●●●○●○	●●	
Functionalization	2018-07-02 11:41	NL-B4B			○●●●○●○	●●	
Sizing	2018-07-02 11:47				○●●●○●○	●●	
Association	2018-07-02 00:11	hCA1	Anti-hCA1		○●●●○●○	●●	
Dissociation	2018-07-02 13:07	hCA1	Anti-hCA1	10 nM	○●●●○●○	●●	
Standby	2018-07-02 13:34	NL-B4B	cNL-A48 ar	500 nM	○●●●○●○	●●	

3. Click on **CREATE NEW ANALYSIS** in the lower right corner and select the **Kinetics** analysis type to analyze the kinetic rates.

Please select an analysis type




4. On the **SETTINGS** page you will see the data you just imported. Association and dissociation should be paired automatically in the main column, or you can drag-and-drop them from the left column {1} into the respective box and add them with the middle button  {2}. The fitting range is preselected automatically as well and indicated by the sliders. Select each measurement one by one in order to verify that the fitting borders are set accurately. They can be adjusted by entering the number after double-clicking on the value or by moving the slider {3}. Proceed by clicking on **FIT ANALYSIS** in the lower right corner.



- You will see a mono-exponential fitting curve for the association and dissociation data. To incorporate all the data, the global mono-exponential fitting model is selected in the left column. One value for k_{on} , k_{off} and K_D is calculated from all curves.
- Next, click on the top next to “Kinetics 1” on the + tab to create a different analysis.



Open the subfolders of **Sizing {1}** by clicking on the triangle. Drag-and-drop the reference DNA (**cNL-A48 and B48**) and all three **hCA1** measurements marked with the Sizing  symbol to the right {2}. Directly **remove all green** recordings since we were only measuring in the red channel. Click on **CREATE NEW ANALYSIS** in the lower right corner and select the **Sizing** analysis type to deduce the size of hCA1.

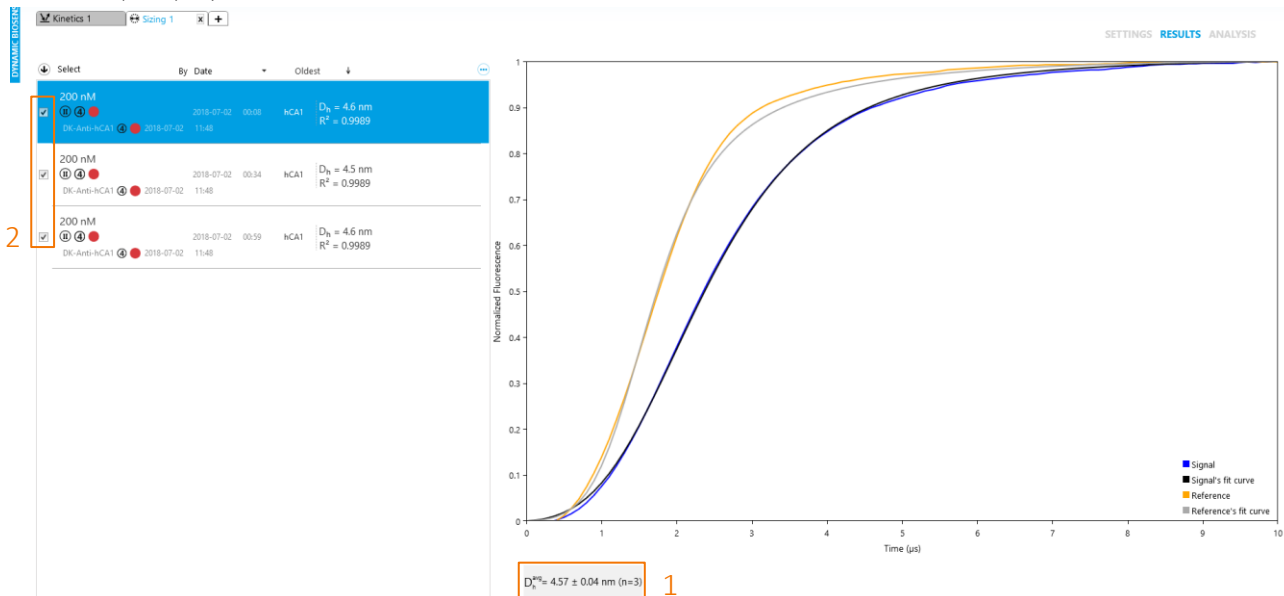
Name	Time	Ligand	Sample	Concentration	Electrode	Color	Type
2018-07-02 11:15-DK-Anti-hCA1	2018-07-02 11:18						
Passivation	2018-07-02 11:18	Air		0 M			
Kinetics	2018-07-02 11:41						
Functionalization	2018-07-02 11:41	NL-B48					
Sizing	2018-07-02 11:47						
cNL-A48 and B48 [500 nM]	2018-07-02 11:47	cNL-A48 a cNL-A48 ar		500 nM			
cNL-A48 and B48 [500 nM]	2018-07-02 11:48	cNL-A48 a cNL-A48 ar		500 nM			
hCA1 [200 nM] (1)	2018-07-02 00:07	hCA1	hCA1	200 nM			
hCA1 [200 nM] (1)	2018-07-02 00:08	hCA1	hCA1	200 nM			
Anti-hCA1 [2.5 nM]	2018-07-02 00:14	hCA1	Anti-hCA1	2.5 nM			
Anti-hCA1 [2.5 nM]	2018-07-02 00:14	hCA1	Anti-hCA1	2.5 nM			
hCA1 [200 nM] (2)	2018-07-02 00:33	hCA1	hCA1	200 nM			
hCA1 [200 nM] (2)	2018-07-02 00:34	hCA1	hCA1	200 nM			
Anti-hCA1 [5 nM]	2018-07-02 00:39	hCA1	Anti-hCA1	5 nM			
Anti-hCA1 [5 nM]	2018-07-02 00:40	hCA1	Anti-hCA1	5 nM			
hCA1 [200 nM] (3)	2018-07-02 00:59	hCA1	hCA1	200 nM			
hCA1 [200 nM] (3)	2018-07-02 00:59	hCA1	hCA1	200 nM			
Anti-hCA1 [10 nM]	2018-07-02 13:05	hCA1	Anti-hCA1	10 nM			
Anti-hCA1 [10 nM]	2018-07-02 13:06	hCA1	Anti-hCA1	10 nM			
Association	2018-07-02 00:11	hCA1	Anti-hCA1				
Dissociation	2018-07-02 13:07	hCA1	Anti-hCA1	10 nM			
Standby	2018-07-02 13:34	NL-B48	cNL-A48 ar	500 nM			

- On the **SETTINGS** page you should first reference your data if not done automatically. To do so, drag-and-drop the **cNL-A48 and B48** measurement on each **hCA1** measurement. If a measurement is referenced, it is indicated by the measurement name and time of the reference {1}. Set the *Running buffer* used (**PE40**) in the options below the graph. Finally click on **FIT ANALYSIS** in the lower right corner.

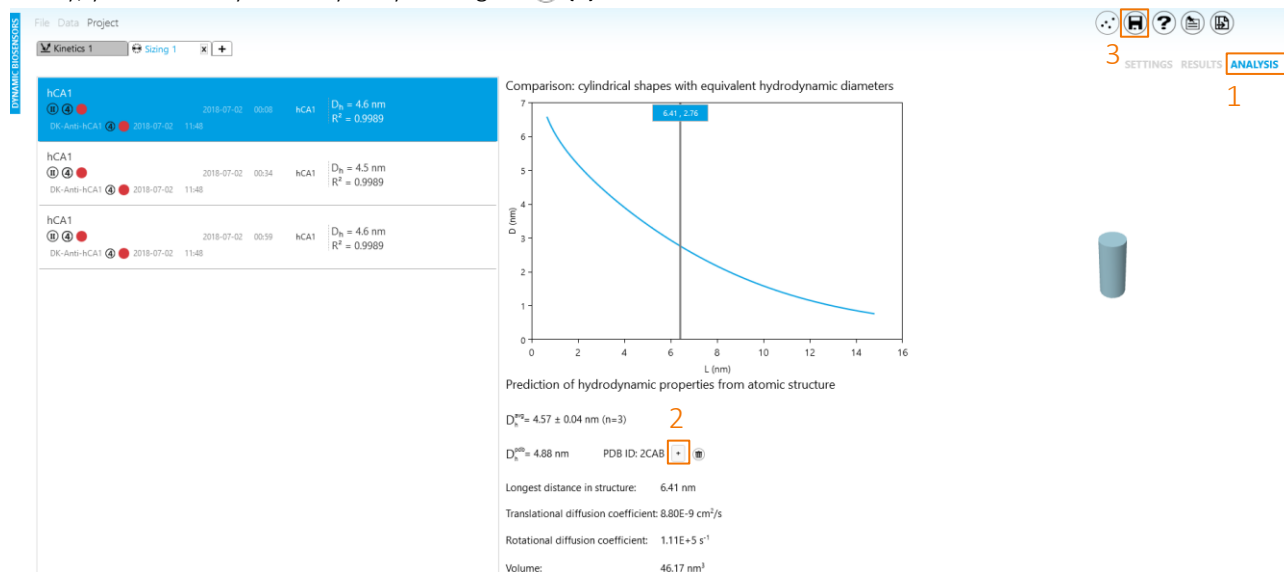


- On the **RESULTS** page a combined plot of the conjugate (hCA1) and reference sizing data is shown. Each sizing consists of four sigmoidal curves: The reference curve is plotted in **orange**, the conjugate in **blue**, and the corresponding fit curves in grey and black, respectively. The left panel lists the calculated hydrodynamic diameter with the coefficient of

determination (R^2) for each individual measurement. Below the graph an *average hydrodynamic diameter* D_h^{avg} {1} of all selected {2} sizing measurements is displayed. If more than one sizing measurement was analyzed, the standard error of the mean (SEM) is provided as well.



9. You can compare your measured effective hydrodynamic radius with the radius calculated from a pdb file. Click on **ANALYSIS** on the top right {1} and **load the pdb file** from a local disc or enter the pdb ID for adding it from the Internet {2}. Finally, you can save your analysis by clicking on {3}.



Congratulations, you are a **switchSENSE®** expert user now!

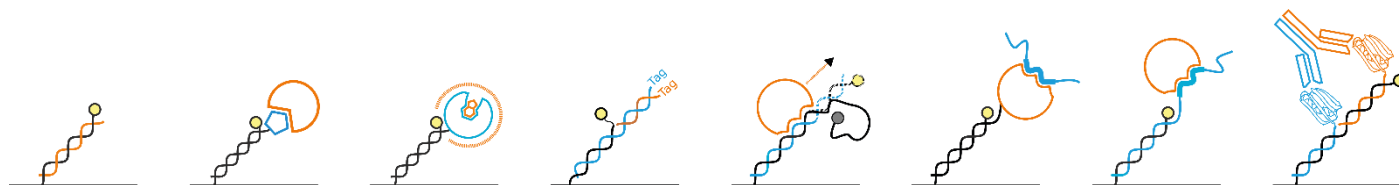
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switchSENSE® is a proprietary measurement technology by Dynamic Biosensors GmbH.

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