

PICO Protein Detection Trial Kit USER MANUAL

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01 Kit Contents and Storage

PICO Protein Detection Trial Kit		
Catalog Number:		PICO-000090
Pouch - store at 4°C	pcs	Item code
Additive T	1	A
Additive C	1	B
Bovine serum albumin	1	C
Prelabeled Trastuzumab antibody (4.82×10^9 cp/μl)	1	D
Prelabeled Pertuzumab antibody (4.82×10^9 cp/μl)	1	E
Pouch - store at -20°C		
Additive L	1	F
Coupling dPCR Mix	1	G
Recombinant human ErbB2/HER2 protein (0.529 μmol/l)	1	H
PICO BL Probe	1	I
PICO P8 Probe	1	J

The PICO Protein Detection Trial Kit is shipped at room temperature. Upon arrival, it is important to ensure that the products are immediately transferred to optimal storage conditions. Please note the different storage temperatures for the components of the PICO Protein Detection Trial Kit. Date of expiry is stated on the back side of the pouches.

02 Intended Use

The PICO Protein Detection Trial Kit is intended for research use only (RUO). The product is not intended for the diagnosis, prevention, or treatment of a disease. All due care and attention should be exercised in the handling of the product. We recommend the users of the Actome products to adhere to the national safety guidelines.

03 Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDS). These are available at: www.actome.de/resources/downloads

In case of reagent spillage, absorb the spilled material, dispose of it accordingly and clean with suitable laboratory detergent and water. If the spilled liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (w/v) sodium hypochlorite.

04 Quality Control

Each lot of PICO Protein Detection Trial Kit is tested against predetermined specifications to ensure consistent product quality.

05 Introduction

Protein Interaction Coupling (PICO) is a highly sensitive immunoassay for the detection and quantification of proteins, protein interactions, and post-translational modifications using a dPCR instrument for readout. The PICO Protein Detection Trial Kit is an ideal tool for researchers who want to become familiar with the PICO assay. It includes all the necessary reagents to perform one PICO measurement, using the recombinant human ErbB2/HER2 protein as the target, along with two pre-labeled antibodies (trastuzumab and pertuzumab) that are directed against ErbB2/HER2. The Trial Kit highlights the unique features of PICO, including ultra-high sensitivity, zero background, wash-free workflow, and high specificity. With the PICO Protein Detection Trial Kit, you'll gain a deep understanding of the core PICO technology, experimental set-up, and data analysis using the [AMULATOR](#). Additionally, the kit comes with a detailed protocol for setting up a calibration curve experiment (CLC), which involves using a fixed antibody concentration and a dilution series of the sample input to demonstrate the relative quantification (RQ) evaluation of the assay (**Figure 1**).

If you would like to design your own PICO assay consider the following instructions:

The PICO assays can be performed using two, three, or four different labeled antibodies, depending on how many targets you would like to measure. A target can be a protein, a protein interaction, or a post-translational modification. For the detection of an individual target two different labeled antibodies are required. In the case of a single protein detection the two antibodies must bind to non-overlapping epitopes. For the detection of a protein interaction one antibody per interacting partner is required. To detect a post-translational modification (PTM) a PTM-specific antibody and an antibody directed against the PTM-bearing protein is required. The molecular complex, consisting of the target and two bound antibodies (called 'couplex'), is the molecular detection unit of the PICO assay.

For the PICO assay the antibodies of your choice are labeled with PICOglue Labels (available in four versions: BL, P8, N6, or O7) using the PICOglue Antibody Labeling Kit. After the antibody labeling, the PICO Amplification Core Kit is required to run the PICO assays.

Day	Steps	Timing	Hands-on	Stopping points & Storage
DAY 1	Step 1 – Preparation of PICO Assay	55 min	15 min	
	A. Preparation of Dilution Series of Protein	30 min	10 min	
	B. Setup of Antibody Mix	25 min	5 min	
	Step 2 – Calibration Curve	4 h	40 min	
DAY 2	A. Binding Reaction	30 min + ON	10 min	● overnight incubation (ON) at 4°C
	B. Dilution of the Samples before dPCR	20 min	20 min	
	C. QIAcuity dPCR Run	3 h 10 min	10 min	● overnight run possible
	Step 3 – Evaluation	35 min	25 min	
	A. Raw Couplex Calculation using AMULATOR	15 min	5 min	
	B. Relative Quantification	20 min	20 min	

Figure 1. The PICO Protein Detection Trial Kit workflow.

06 Scientific Background of a CLC Experiment

06.1 Explanation of the Bell-shaped Curve

The Protein Detection Trial Kit protocol includes a calibration curve (CLC) experiment to determine the optimal linear range between antibody and target concentration. In the protocol a dilution series of the biological sample with a constant antibody concentration is prepared. If the antigen concentration in the sample is high enough, the couplex counts derived from a CLC experiment can form a bell-shaped curve when plotted against the dilution series of the sample input (**Figure 2**).

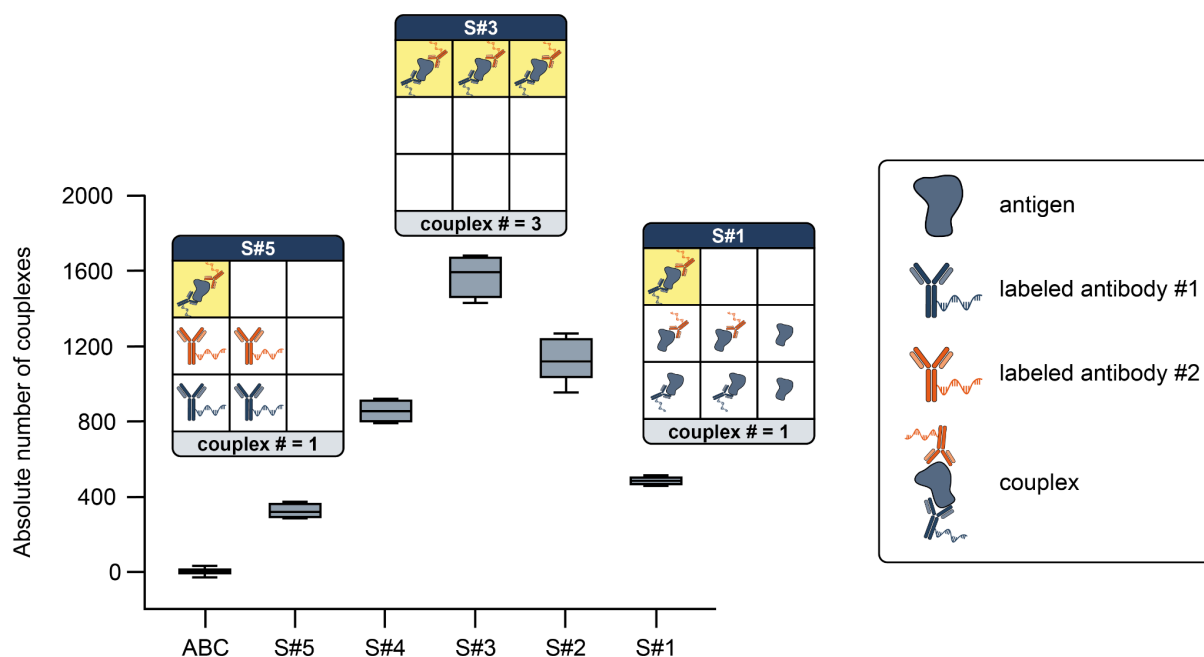


Figure 2. Typical result of measuring a dilution series with PICO: a bell-shaped curve

Boxplots representing the absolute number of couplexes detected at different dilution steps (from highest 'S#5' to lowest 'S#1' dilution). The pattern of the boxplots resembles a bell-shaped curve. Ratios between antigen and labeled antibodies are depicted at three different dilution steps (S#5, S#3, and S#1). Couplexes are highlighted with a yellow background. The central solid line within each box is the mean, and the bottom and top of each box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles. ABC - antibody control

Diluting the biological sample leads to a decreasing amount of antigen in the binding reaction (the mixture of biological sample and labeled antibodies). In **Figure 2** the absolute number of couplexes, measured from a 3-fold dilution series of recombinant HER2 protein using 4×10^{-11} M antibody concentration in the binding reaction, is depicted. The S#1 sample contains the highest antigen amount (1×10^{-9} M), while the S#5 sample has the lowest antigen amount (1.23×10^{-11} M). The Antibody Control (ABC) is a control sample containing only the labeled antibodies.

On the left side of the curve (low antigen side), the ratio of antibody to antigen is high, leading to decreased probability of couplex detection (S#5 to S#4 in **Figure 2**). The high dilution of the sample leads to a loss of couplex count in the dPCR, since the couplexes are diluted out and the compartments are occupied mainly by unbound labeled antibodies.

Increasing the antigen concentration leads to an increasing probability of couplex formation. In a certain range, this trend is linear until an equilibrium between antigen and antibodies is

reached (approximately at S#3 in **Figure 2**). At this equilibrium, the highest amount of complexes are measured.

On the right side of the curve (high antigen side), the further increase in antigen concentration leads to a decrease in complex count (S#2 to S#1 in **Figure 2**). The increase of antigen amount decreases the chance of complex formation, since the chance that the two different labeled antibodies bind to the same target is smaller. In a certain range this trend is linear until the number of partitions of the dPCR is too low for complex detection.

The linear correlation between the complex counts and the antigen amount at the low antigen side of the curve (S#5 to S#4 in **Figure 2**) can be used for relative quantification (RQ), to relatively compare the amount of target between different sample types. For this, the raw complex counts are processed and the molar ratio between the complex counts of two different samples is calculated (see 10.2 and 10.3). The normalization of sample input allows the comparison of data derived from different amounts of sample. However, for relative comparison of two samples, the same antibody concentration in the binding reaction has to be used. Additionally, for better comparability between experiments, we recommend to use the same batch of labeled antibodies.

06.2 Using CLC Experiments for Relative Quantification in Different Biological Samples

Careful planning of an CLC experiment is necessary to generate a dataset that can be used for relative quantification. See the different outcome scenarios for a CLC experiment in **Figure 3**.

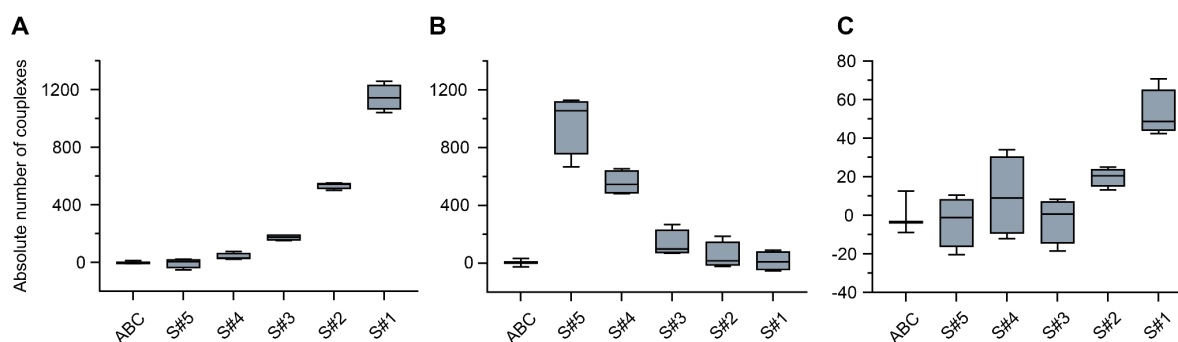


Figure 3. Hypothetical outcome scenarios from a calibration curve (CLC) experiment.

Boxplots representing the absolute number of complexes detected at different dilution steps (from highest 'S#5' to lowest 'S#1' dilution). (A) PICO data reflecting the low antigen side of the bell-shaped curve, indicated by increasing complex count with increasing antigen concentration. (B) PICO data reflecting the high antigen side of the bell-shaped curve, indicated by decreasing complex count with increasing antigen concentration. (C) PICO data with low complex counts due to over diluted samples or low expression levels of the measured target. The central solid line within each box is the mean, and the bottom and top of each box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles. ABC - antibody control.

In general, biological samples with a high expected concentration of the measured target require less sample input in the binding reaction, while biological samples with a low concentration of the measured target require more sample input. For initial experiments we recommend starting from a cell lysate equivalent of 20,000 cells in the binding reaction and performing a 2-fold (low expressing target) or 3-fold (high-expressing target) dilution series. Since biological samples vary widely, these settings may need to be adjusted for each sample. In case of using recombinant protein material, we recommend to perform a dilution series where the concentration of the dilution step S#3 is in the same range as the antibody concentration used in the binding reaction (in most cases 4×10^{-11} M).

In an optimal case, a CLC experiment results in a curve as depicted in **Figure 3A**. In this example the antibody concentration in the binding reaction is 4×10^{-11} M. The S#1 sample contains the highest protein amount (4×10^{-11} M) while S#5 has the lowest protein amount (4.94×10^{-11} M). Increasing the sample input leads to an increase in complex count, indicating that the measured data is located on the low antigen side of the bell-shaped curve. This CLC pattern is observed for most experimental setups, especially if the expression of the target molecule is rather low. Data points derived from dilutions in the linear range of this curve can be used for RQ. We recommend not to use the data from the highest measured complex count to ensure that only data from the linear range is used.

In another scenario, a CLC experiment might result in a curve as depicted in **Figure 3B**. In this example the antibody concentration in the binding reaction is 4×10^{-11} M. The S#1 sample contains the highest protein amount (3×10^{-8} M) while S#5 has the lowest protein amount (1.23×10^{-10} M). Increasing the sample input leads to a decrease in complex count, indicating that the measured data is located on the high antigen side of the bell-shaped curve. This CLC pattern is observed for experimental setups with a high expression level of the target. In this case, we recommend repeating a CLC experiment with higher dilution factors for the biological samples so that the results reflect complex counts as seen in **Figure 3A**. However, this scenario will be observed only with very highly expressed targets.

In a third scenario, a CLC experiment might result in a curve as depicted in **Figure 3C**. In this example the antibody concentration in the binding reaction is 4×10^{-11} M. The S#1 sample contains the highest protein amount (1.27×10^{-12} M) while S#5 has the lowest protein amount (1.57×10^{-14} M). Very low complex counts are measured for all dilution steps. In this case, the expression level of the target might be too low for the chosen experimental setup. We recommend increasing the overall sample input (e.g. using a higher amount of lysed cells) or decreasing the dilution factors for the biological sample.

The CLC experiment described in the PICO Protein Detection Trial Kit protocol results in a similar curve as shown in **Figure 2**. It demonstrates the full bell-shaped curve at the different antigen-to-antibodies ratios.

07 Preparation for PICO Assay

07.1 General Remarks

- The PICO assay is sensitive to variations in pipetting. Make sure all pipettes are calibrated for reliable liquid handling. The dilution factor for dPCR is usually very high and it is important to use highest pipetting precision available to ensure reliable quantitative results (see recommended equipment and devices in *07 Equipment and Reagents to be Supplied by User*).
- Use gentle mixing by pipetting up and down. This helps to reduce spill-overs and loss of material.
- Change gloves regularly during laboratory work to avoid contamination. Contamination can be detected if the NTC sample shows positive partitions in any color.
- To ensure the highest molecular sensitivity and reproducibility of PICO we recommend using four technical replicates of each sample.
- In addition to the samples and the non template control (NTC for dPCR), antibody control (ABC) reactions are also set up. An ABC reaction contains the corresponding antibody mix without the sample. In these samples, the two different labeled antibodies colocalize in the compartments only according to Poisson statistics. Therefore, the couplex count in the ABC should be zero. However, due to offsets in the dPCR (e.g. signal dropouts or incorrect clustering) the ABC value may deviate from zero. Thus, the AMULATOR performs an automatic ABC normalization by default.
- The average number of targets per partition is called lambda. Lambda is also a statistical parameter for the underlying Poisson distribution. A higher lambda value means higher assay sensitivity, but also a larger standard deviation based on Poisson statistics and a loss of signal due to dropouts, since several templates have to be amplified in one partition. For the PICO assay, we recommend a lambda of 0.15, however any lambda values between 0.01 and 0.6 can be used for PICO.
- The sensitivity of the assay is also influenced by the lambda values. For a low expressing target, the sensitivity of the assay at low lambda values might be too low and the assay should be repeated with optimized settings.
- To reach an optimal lambda for the PICO assay, the samples must be diluted prior to the dPCR. The dilution of the samples prior to dPCR disrupts the equilibrium binding conditions and induces dissociation of the antibodies from their target. After the samples have been diluted, make sure to work without interruption until the QIAcuity Nanoplate is loaded into the QIAcuity.
- Dilutions of antibody stocks, used in the binding reaction, can be stored for up to 12 months at 4°C if prepared in 1x PICOglue Storage Buffer from the PICO gAL Kit. Please consider that highly diluted antibodies (larger than 200-500-fold diluted stocks) might have decreased stability. Therefore, for storage, an intermediate dilution step (20-200 fold) in 1x PICOglue Storage Buffer is recommended.
- Please consider that the sensitivity of the assay is dependent on the amount of partitions. If using the QIAcuity Nanoplate 8.5k 96-well instead of the QIAcuity Nanoplate 26k 24-well, the sensitivity is reduced by about an estimated factor of 3.



07.2 Plate Layout of Binding Reaction and dPCR Pre-Dilution Setup

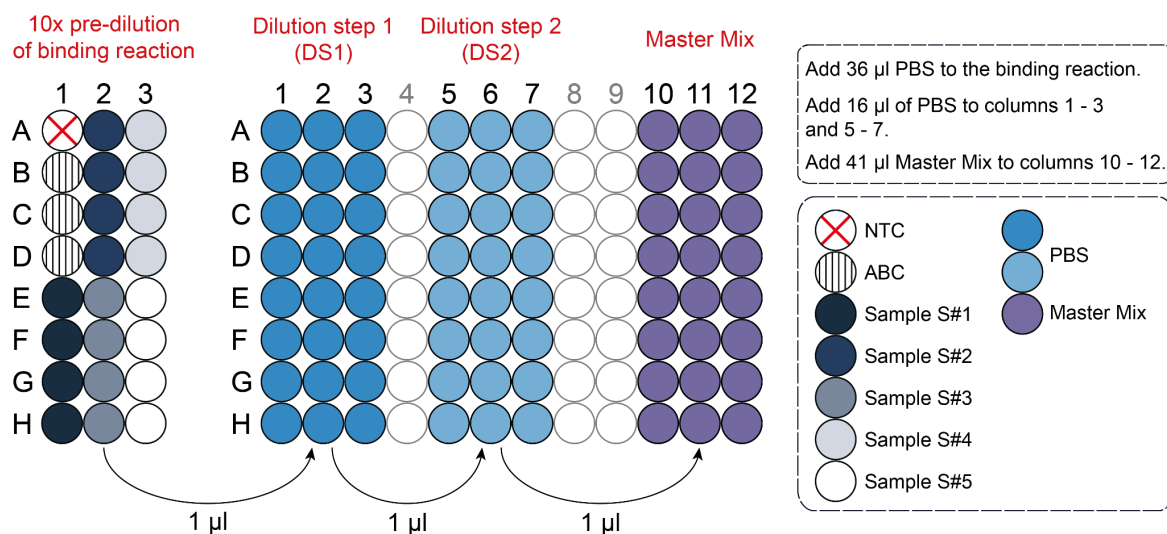


Figure 4. Plate layout of the binding reaction and the dPCR pre-dilution plate setup. Here, three replicates for the ABC and four replicates for the samples are prepared. The dPCR pre-dilution is performed in two steps. ABC - antibody control; NTC - non-template control.

The binding reaction setup and the dPCR pre-dilution for the PICO Trial Kit assay can be seen in **Figure 4**. First the dilution series of the sample is combined with the antibody mix and incubated overnight (binding reaction). We recommend preparing three technical replicates for the ABC and four technical replicates for each sample from the binding assay. The following day the binding reaction is diluted for the dPCR reaction. First, a 96-well plate is prepared by adding 16 µl of PBS to columns 1 - 3 and 5 - 7, and 41 µl of Master Mix to column 10 - 12. Then, the binding reaction is diluted 1:10 using PBS and mixed by pipetting 30 times ('pre-dilution of the binding reaction'). From each diluted binding reaction sample, 1 µl is transferred into the corresponding wells of the first dilution step (DS1) and the dilution is mixed by pipetting 30 times. Then, 1 µl of DS1 is transferred to the corresponding wells of the second dilution step (DS2) and the dilution is mixed by pipetting 30 times. Finally 1 µl of DS2 is transferred to the wells containing the Master Mix. After mixing by pipetting three times, 40 µl of the Master Mix containing the diluted sample are transferred to a QIAcuity Nanoplate 26k 24-well and dPCR is performed. Perform the dilution steps as fast as possible since the dilution disrupts the equilibrium binding conditions and induces antibody dissociation.

08 Equipment and Reagents to be Supplied by User

Notes

Devices/Equipment

- QIAcuity Digital PCR System (QIAGEN, Cat.#: 911001)
- Table-top mini centrifuge for quick spins (~1,000 rcf)
- Plate centrifuge (e.g. Megafuge 8, Thermo Fisher, Cat.#: 75007210)
- Vortex mixer
- Ultrasonic bath
- Multichannel pipette, 8-channel (1 – 10 µl)
- Multichannel pipette, 8-channel (10 – 100 µl)
- Regular 1-channel pipettes (1 – 1,000 µl)
- Electronic Multichannel pipette (INTEGRA, Cat.#: 4722), 12-channel, 5 – 12.5 µl (optional)

Consumables

- QIAcuity Nanoplate 26k 24-well (QIAGEN, Cat.#: 250001)
- 2 PCR microplates, 96 well, Polypropylen, V-bottom or similar
- Sealing foil Adhesive Film (e.g. Thermo Fisher, Cat.#: 10696771)
- 12.5 µl GRIPTIP, Sterile, Filter 5 Racks of 384 Tips, long; for electronic multichannel pipette (INTEGRA, Cat.#: 3405) (optional)
- 1.5 ml reaction tubes
- 0.5 ml reaction tubes
- 10 µl, 200 µl, 1,000 µl standard pipette tips

Chemicals and Kits

- QIAcuity Probe PCR Kit (QIAGEN, Cat.#: 250101)
- Phosphate-Buffered Solution (PBS), without calcium or magnesium ions (e.g. Thermo Fisher Scientific, Cat.#: 12037539)
- cOmplete Protease™ Inhibitor Cocktail (Roche, Cat.#: 04693132001)
- Ultrapure water (e.g. Thermo Fisher Scientific, Cat.#: 15667708)



09 Protocol of Protein Detection Trial Kit

Note: The protocol is valid for performing a single PICO assay using the PICO Protein Detection Trial Kit.

09.1 Buffer Preparation

1. Prepare the chemicals and buffers as listed below.
Prepare the buffers directly before they are used.

Additive C (5x stock)

Add 500 μ l PBS

Easily soluble, stable for 1 week at 4°C.

BSA (5x stock)

Add 400 μ l PBS

Stable for 3 days at 4°C

EDTA-free Protease Inhibitor Cocktail (PIC), (25x stock)

1 tablet of cOmplete ProteaseTM Inhibitor Cocktail

2 ml PBS

Stable for 12 weeks at -20°C

Lysis Buffer Stock (LB-Stock), (2x stock)

200 μ l Additive T

400 μ l Additive C

80 μ l PIC

200 μ l Additive L

120 μ l PBS

Stable for 3 days at 4°C

Lysis Buffer (LB)

300 μ l LB-Stock

300 μ l PBS

Stable for 3 days at 4°C

Control Buffer (CB)

250 μ l LB-Stock

100 μ l BSA

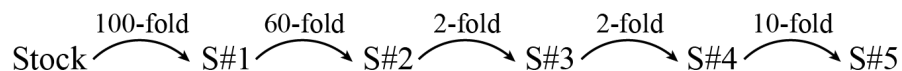
150 μ l PBS

Prepare the Control Buffer fresh



09.2 Preparation of the Dilution Series

2. Thaw a vial of the supplied recombinant human ErbB2/HER2 protein and spin it down (~1,000 rcf, 5 s).
3. Prepare a dilution series of the protein in LB:



Start with a 100-fold dilution (sample S#1). Next, dilute sample S#1 60-fold (total dilution factor: 6,000) to generate sample S#2. Dilute sample S#2 2-fold (total dilution factor: 12,000) to generate sample S#3. Dilute sample S#3 2-fold (total dilution factor: 24,000) to generate sample S#4. Finally, dilute sample S#4 10-fold (total dilution factor: 240,000) to generate sample S#5. Prepare a volume of at least 60 µl for each sample. If required, plan the dilution series using the [PICO Calculator](#) (Step 1 in the 'PICO Calculator' tab).

09.3 Binding Reaction

4. Prepare 100 µl of antibody mix (ABX) by adding 1 µl of each antibody (PTZ and TTZ) to 50 µl of LB-Stock and 48 µl of PBS. You can also calculate the required volume of antibodies, LB-Stock, and PBS by using the [PICO Calculator](#) (Step 2 in the 'PICO Calculator' tab). To calculate the ABX manually check *section 11.1 Calculation of the ABX Mix*.
5. Set up the binding reaction in a 96-well PCR microplate (v-bottom) (**Figure 4**). For the NTC, add 4 µl of LB to one dedicated well.
6. For the ABC, combine 2 µl of CB with 2 µl of ABX and make three technical replicates.
Due to the small volume of the binding reaction, do not mix by pipetting.
7. For the biological samples, combine 2 µl of the ABX with 2 µl of S#1 to S#5. Make four technical replicates of each dilution step.
8. Seal the plate (without the lid) with an adhesive foil. Sonicate the plate at full power for 1 min in an ultrasonic bath. Centrifuge the plate to collect the liquid at the bottom (~1,000 rcf, 30 s) and incubate at 4°C overnight.
The incubation time can be varied between 12-24 hours.



09.4 Pre-Dilution and Digital PCR

9. Prepare the Master Mix for 24 samples (see amounts below). Vortex for 10 s and spin down (~1,000 rcf, 5 s).
Store the Master Mix at 4°C.

Master Mix	
Reagents	Volume (µl)
Ultrapure water	697
QIAcuity Probe Master Mix	284
PICO Probe P8	45
PICO Probe BL	45
Coupling dPCR Mix	36

10. Calculate the dilution factor to reach the optimal lambda (0.15) for the dPCR using the [PICO Calculator](#) (Step 3 in the 'PICO Calculator' tab). To calculate the dilution series manually follow the steps in *section 11.2 Calculation of the Dilution Factor*.
Make sure to select Nanoplate 26k 24-well in the PICO Calculator. Here in the Trial Kit User Manual, the required carry-over volume, dilution steps, and the dilution volume is already calculated.
11. Prepare a 96-well plate with columns 1 - 3 and 5 - 7 containing 16 µl of PBS and columns 10 - 12 containing 41 µl of Master Mix (**Figure 4**).
12. Remove the adhesive foil from the plate containing the binding reaction carefully. Add 36 µl of PBS to the wells (represents the first 10x pre-dilution). Mix thoroughly by pipetting up and down 30 times.
Perform the following steps without unnecessary breaks as the dilution disrupts the equilibrium binding conditions and induces antibody dissociation.
13. Transfer 1 µl from each pre-diluted sample into the corresponding wells (columns 1 - 3) of the dilution plate. Mix by pipetting up and down 30 times (**Figure 4**).
This dilution step is defined as DS1.
14. Transfer 1 µl from the first dilution step (DS1) into the next set of corresponding wells (columns 5 - 7). Mix by pipetting up and down 30 times (**Figure 4**).
This dilution step is defined as DS2.
15. Transfer 1 µl from the last dilution step (DS2) into the next set of corresponding wells containing 41 µl Master Mix (columns 10 - 12). Mix by pipetting up and down 30 times (**Figure 4**).



16. Finally transfer 40 µl of the Master Mix containing the diluted samples into a QIAcuity Nanoplate 26k 24-well. Seal the plate according to the QIAcuity user manual protocol and insert the plate into the QIAcuity dPCR System. Run the dPCR program on the QIAcuity according to the instructions of the manufacturer using the following dPCR settings:

Priming			
QIAGEN Standard Priming Profile			
PCR conditions			
Step	Temperature	Time	Cycle 40 times
Hot-start	95°C	2 min	
Denaturing	95°C	15 s	
Annealing	58°C	30 s	

Imaging conditions			
PICO Probe	QIAcuity Channel	Integration time	Gain
P8 Probe	FAM, green channel	500 ms	6
BL Probe	HEX, yellow channel	400 ms	6



10 Evaluation

10.1 Raw Couplex Calculation using AMULATOR

17. After the dPCR run, using the QIAcuity Software Suite, select your plate in the 'Plates Overview', click 'Analyze' and select all wells. In the 'Select targets' drop down window click 'Select All' and press 'Show results'.
18. Select '1D Scatterplot' in the menu above the results and adjust the thresholds in all channels. We recommend placing the threshold close to the negative population. Please note that directly after adjusting the threshold for a channel, the values have to be recalculated.
19. After adjusting the thresholds, download the raw dPCR data (RFU values). For this, select 'List', click 'Export to CSV...' and select 'RFU values'. The download generates a zip file containing csv files (one for each fluorescent color). Unzip the exported file.
Please control the raw data and images of the plate in the QIAcuity Software Suite to ensure that the data is valid and the experimental setup was performed correctly.
20. Prepare the [Sample Definition File](#). The spreadsheet contains two tabs: 'Layout' and 'Settings'. In the 'Layout' tab fill in the name of the samples ('SampleName') to the corresponding wells. Replicates are defined by having the same sample name. In the 'ABC' column mark the ABC samples with 'true'. In the 'ExperimentalGroup' column, group together the samples with their corresponding ABC control by using the same number. If the same ABX is used for all samples, use the same number. The NTC sample is in its own group. In the 'Layout' column choose the plate type ('QIA24' for QIAcuity Nanoplate 26k 24-well or 'QIA96' for QIAcuity Nanoplate 8.5k 96-well.)
Important: Sample names should contain letters (e.g. sample #1). In case of invalid wells (wells containing no signals) the corresponding wells in the sample definition file as well as the according table line have to be deleted to create a continuous table.
21. In the 'Settings' tab of the sample definition file use the dropdown menu to assign the labeled antibodies to the correct fluorescent color according to the PICO labels used (HEX—BL, FAM—P8, TAMRA—N6, ROX—O7).
22. The [AMULATOR](#) can be accessed here: <https://amulator.actome.de/>
23. First-time users require an initial registration to our server. Registration provides a personal cloud-based storage space, where you can store and (re)analyze your uploaded PICO datasets (up to 15 files). The files are only accessible by you.
24. Create a project and upload the Sample Definition File as well as the unzipped single raw RFU files using the ten-character token provided with the kit. The projects are organized in a table. By clicking on the delete sign you can permanently delete them (after confirmation).



25. Select the project you would like to analyze and click on 'Calculate Results' to initiate the data analysis.
26. AMULATOR creates two graphs in the Results tab. The complex chart displays the number of complexes for each sample and each antibody pair. The lambda charts represent the lambda values of each antibody in each well. The individual boxplots can be rearranged by drag and drop using the sample name.
27. To download the results click on the 'Export data' button. To download the graphs click on the 'Export graph images'.
28. To perform the Relative Quantification of the data, follow the steps in *section 11.3 to 11.4*.



11 Appendix - Calculations

11.1 Calculation of the ABX Mix

1. To calculate the volume of each antibody stock added to the ABX, the concentration of the antibody stocks must be converted from copies per μl to molar concentration.

$$C_M = C_C \times 10^6 / N_A$$

C_M : Conc. of antibody stock [M]

C_C : Conc. of antibody stock [cp/ μl]

This concentration is determined via dPCR in the ABQC

N_A : Avogadro constant ($6.02214076 \times 10^{23} \text{ mol}^{-1}$)

Example: The concentration of the supplied antibody stock solutions (C_C) is $4.82 \times 10^9 \text{ cp}/\mu\text{l}$. The calculated molar concentration is ($8 \times 10^{-9} \text{ M}$).

2. Calculate the volume of each antibody (V_{AB1} or V_{AB2}) used in the ABX based on the following calculation.

If V_{AB1-2} are too low for precise pipetting, prepare a pre-dilution of the antibody stock using LB. In this case, the concentration of the pre-dilution of the antibody (DF_{AB}) has to be considered in the calculation.

$$V_{AB} = V_{ABX} \times C_{BRM} \times DF_{BR} \times DF_{AB} / C_M$$

V_{AB} : Volume of antibody stock (or dilution) added to the ABX

V_{ABX} : Total volume of ABX; we recommend at least 60 μl

C_{BRM} : Conc. of antibody in the binding reaction [M];

we recommend using $4 \times 10^{-11} \text{ M}$

DF_{BR} : Dilution factor introduced by pipetting of binding reaction

DF_{AB} : Dilution factor introduced by pre-dilution of antibody stock;

use '1' if no predilution is performed

Example: The molar concentration of the antibody stock is $4.82 \times 10^{-9} \text{ M}$. We choose 100 μl as the total volume of the ABX and an antibody concentration of $4 \times 10^{-11} \text{ M}$ in the binding reaction. The dilution factor introduced by the binding reaction setup is 2 in our case (2 μl of the ABX is combined with 2 μl of the cell lysate). The calculated volume of each antibody stock added to the ABX is 1 μl .

3. Prepare the ABX using LB-Stock. Add the calculated volume of the two antibody stock solutions or prepared dilutions and fill the remaining volume with PBS.

If a high volume of antibodies has to be used, the amount of PBS added to the ABX might be negative, in this case use LB to fill up the whole ABX volume.

Example: The total volume of the ABX is 100 μl . Add 50 μl of LB-Stock in a low protein binding tube. The volume of each antibody added to the ABX is 1 μl . Add 48 μl PBS to fill up the volume to 100 μl .



11.2 Calculation of the Dilution Factor

- Convert the antibody concentration, used in the binding reaction, from molar concentration to copies per μl .

$$C_{BRC} = C_{BRM} \times N_A / 10^6$$

C_{BRC} : Conc. of antibody in the binding reaction [$\text{cp}/\mu\text{l}$]

C_{BRM} : Conc. of antibody in the binding reaction [M]

N_A : Avogadro constant ($6.02214076 \times 10^{23} \text{mol}^{-1}$)

Example: The concentration of antibodies in the binding reaction is $4 \times 10^{-11} M$. The concentration in $\text{cp}/\mu\text{l}$ is 2.4×10^7 .

- Calculate the targeted antibody concentration for the dPCR.

$$C_d = \lambda / V_p$$

C_d : Targeted conc. of antibodies for the dPCR [$\text{cp}/\mu\text{l}$]

λ : Average number of targets per partition:
we recommend 0.15

V_p : Volume of a partition [μl]: 0.00078 μl

Example: We choose a lambda of 0.15. The volume of one partition of a QIAcuity Nanoplate 26k 24-well is 0.00078 μl . Thus, the targeted concentration for the dPCR is 192.31 $\text{cp}/\mu\text{l}$.

- Calculate the dilution factor necessary to reach an optimal lambda range for the dPCR (DF_d). Based on the small volume of the binding reaction, we recommend to perform a pre-dilution of the binding reaction. This, together with the dilution introduced by the volume of the Master Mix has to be considered in the calculation of the DF_d .

$$DF_d = C_{BRC} / (C_d \times DF_{pre} \times DF_{MM})$$

DF_d : Dilution factor necessary to reach an optimal lambda

DF_{pre} : Dilution factor of pre-dilution; we recommend 10

DF_{MM} : Dilution factor introduced by Master Mix; usually 42

Example: The concentration of antibodies in the binding reaction is 2.4×10^7 copies/ μl and the targeted concentration for the dPCR is 192.31 copies/ μl . Considering a 10x pre-dilution of the binding reaction and the dilution introduced by the volume of the Master Mix, the dilution factor is 298.24 (which can be rounded to 298). This means 1 μl of the 10x pre-diluted sample is diluted in 297 μl PBS.



- Normally, the necessary dilution volume to reach the optimal lambda range is very high. For easier handling we recommend to perform a serial dilution using the following calculation.

Please consider that the V_d is rounded for easier handling of the pipetting.

$$V_d = (((DF_d)^{\frac{1}{\#DS}}) \times V - V_c)$$

V_d : Volume [μl] of PBS required for each dilution step

#DS: Number of dilution steps

V_c : Carry-over volume of previous dilution; usually 1 μl

Example: The dilution factor corrected for the 10x pre-dilution and the dilution introduced by the volume of the Master Mix is 298.24 (which can be rounded to 298). To prevent using a large dilution volume we chose a serial dilution including two dilution steps (#DS = 2) and a carry-over volume of the previous dilution of 1 μl. This results in a volume of 16.27 μl PBS which is rounded to 16 μl. This means, 1 μl of the 10x pre-diluted binding reaction samples are transferred to 16 μl of PBS for the first dilution step and 1 μl of this dilution step will be transferred to 16 μl of PBS for the second dilution step.

11.3 Couplex Count Processing for Relative Quantification (RQ)

Note: The experimental setup of the PICO Protein Detection Trial Kit allow us to perform RQ and relatively compare the amount of recombinant human ErbB2/HER2 protein in the different sample dilutions on the low antigen side (see *02 Scientific Background of a CLC Experiment*). To demonstrate the bell-shaped curve behavior of the PICO assay, the dilution series in the PICO Protein Detection Trial Kit is set in a manner that S#2 to S#5 are on the low antigen side, while S#1 is on the high antigen side. Therefore, we can use S#2 (6000-fold diluted sample), S#3 (12,000-fold diluted sample), S#4 (24,000-fold diluted sample) and S#5 (240,000-fold diluted sample) for RQ. Sample S#5 is over-diluted to demonstrate the lower detection limit of the assay and it is a statistically non-significant signal above zero. This will be demonstrated mathematically at *section 10.3 step 45*.

- The raw couplexes from the AMULATOR are used as an input for RQ. Download the raw couplex count **without ABC correction** from the AMULATOR software (see *10.1 step 27*).
- Download the 'Current Results' csv file from the QIAcuity Software Suite.

11.3.1 Lambda Normalization

- The antibody concentration in each well of a QIAcuity Nanoplate 26k 24-well is reflected by the lambda value. The lambda values (in particular the distribution of the lambda values of one sample group) serves as a marker for pipetting precision. Normalizing the raw couplex counts based on their lambda values can compensate for pipetting inaccuracy.

Please note that lambda normalization can not completely compensate for pipetting inaccuracy and not for all cases of pipetting inaccuracy, therefore precise pipetting is important to obtain reliable results.



11. Calculate the lambda (λ) (average number of detected targets per partition) of each replicate (from each well of a QIAcuity Nanoplate 26k 24-well) using the 'Current Results' file.

Note: The lambda normalization for the raw couplexes can only be applied for one color. By default, we advise to apply the lambda normalization of the first color of the couplex pair as given by the AMULATOR export (column A) as 'type' informs about the color combination of the couplex. For example 'GY' stands for Green-Yellow. In this case, the lambda normalization will be performed using the lambda values of the green channel.

$$\lambda = \ln\left(\frac{\text{valid partitions}}{\text{negative partitions}}\right)$$

Example: Calculate the lambda values for S#2 (wells A2-D2), S#3 (wells E2-H2), S#4 (A3-D3) and S#5 (wells E3-H3). As an example, the lambda normalization and ABC correction for well A2 is performed. The number of valid partitions for well A2 is 25,427. The number of negative partitions for the green channel is 21,025. The lambda value for this sample for the green channel is 0.190.

12. The Lambda Normalization Factor (NF_{λ}) is calculated for each sample (always for the color given first in the AMULATOR).

$$NF_{\lambda} = \frac{\lambda_{\text{replicate}} - \text{avg}(\lambda_{\text{sample}})}{\text{avg}(\lambda_{\text{sample}})}$$

NF_{λ} : Lambda Normalization Factor for one replicate in the sample

$\lambda_{\text{replicate}}$: Lambda value of the replicate

$\text{avg}(\lambda_{\text{sample}})$: Average of lambda values of the sample

Example: The lambda value for the replicate in well A2 for the green channel is 0.190. The lambda values of the three other replicates for S#3 of wells B2, C2 and D2 for the green channel are 0.168, 0.191 and 0.156. The average of the lambda values is 0.176. The Lambda Normalization Factor for the sample in well E2 for the green channel is 0.078.

13. To calculate the lambda normalized couplex counts, first, the average raw couplex count of a sample has to be calculated ($\text{avg}(\xi_{\text{sample}})$). Then, the absolute value of the average has to be calculated ($|\text{avg}(\xi_{\text{sample}})|$).

Example: The raw couplex count for this replicate is 489. The raw couplex counts for the replicates in well B2, C2 and D2 are 470, 526 and 498. The $|\text{avg}(\xi_{\text{sample}})|$ is 495.75.

14. Calculate the lambda normalized couplex counts (ξ_{λ}) for each replicate using the raw couplex counts from the AMULATOR export.

Please note that the calculation of the lambda normalized couplexes does not influence the mean of the couplex count of a sample group but reduces the standard deviation.

$$\xi_{\lambda} = \xi - (|\text{avg}(\xi_{\text{sample}})| \times NF_{\lambda})$$

ξ_{λ} : Lambda normalized couplex count of one replicate



ξ : Raw couplex count of the replicate

$|avg(\xi_{sample})|$: Absolute value of the average raw couplex counts of the sample

NF_{λ} : Lambda Normalization Factor for the replicate

Example: The Lambda Normalization Factor for the replicate in well A2 for the green channel is 0.078. The raw couplex count for this sample is 489. $|avg(\xi_{sample})|$ is 495.75. The lambda normalized couplex count for well A2 is 450.183.

11.3.2 ABC Correction

15. In the next step the lambda normalized couplex counts will be corrected to the ABC. In an ABC reaction, containing only the corresponding antibody mix without the sample, the antibodies colocalize in the compartments only according to Poisson statistics. Therefore, the couplex count in the ABC should be zero. However, due to offsets in the dPCR (e.g. signal dropouts or incorrect clustering) the ABC value may deviate from zero.

Note: ABC correction should only be applied when the ξ_{ABC} values derive statistically significant from zero, which is checked with a t-test.

Example: The raw couplex counts for the ABC control sample are -50, -45 and -85. The values derive statistically significant from zero (p-value: 0.0413).

16. First, the ABC Correction Factor (CF_A) for an experiment is calculated as the average of the raw couplex count of the ABC control sample.

$$CF_A = avg(\xi_{ABC})$$

ξ_{ABC} : Raw couplex counts of ABC sample

CF_A : ABC Correction Factor

Example: The raw couplex counts for the ABC control sample are -50, -45 and -85. The ABC Correction Factor, the average value of the ABC couplex counts, is -60.

17. The ABC corrected couplex counts (ξ_A) for each replicate is calculated using the lambda normalized couplex counts (see 10.2.1 step 41) and the ABC Correction Factor.

$$\xi_A = \xi_{\lambda} - CF_A$$

ξ_A : ABC corrected and lambda normalized couplex count of one replicate

ξ_{λ} : Lambda normalized couplex count of the replicate

CF_A : Average couplex count of ABC control sample replicates

Example: The lambda normalized couplex count for the replicate in well A2 is 450.183. The ABC corrected, lambda normalized couplex count (ξ_A) for the replicate in well A2 is 510.183.



11.4 Relative Quantification (RQ)

18. For relative quantification ensure to use complex counts from the low antigen side of the curve within the linear range. RQ is usually applied to relatively comparing target amounts in different sample types. In the case of RQ demonstration in this PICO Protein Detection Trial Kit, RQ is applied on samples within one dilution series which can also be used in other experimental setups to verify the data quality of a dilution series.

At the low antigen side of the CLC curve, the raw complex count is decreasing for increasing dilution of the sample and therefore the measured target (see *06 Scientific Background of a CLC Experiment*). In the experimental setup of the PICO Protein Detection Trial Kit, this corresponds to S#2 and S#5.

19. First, test if the lambda-normalized and ABC-corrected complex counts (ξ_A) are within the measurable range of the assay and not below the detection limit by testing if they ξ_A values of a sample are statistically significant different from zero.

Example: The ξ_A values for S#2 (510.18, 552.15, 545.35, 615.60), S#3 (382.45, 309.67, 268.40, 278.14), S#4 (176.25, 205.98, 117.74, 220.02) and S#5 (-30.40, 21.56, -1.63, 94.46) are used to test if the measured dilutions of the recombinant human ErbB2/HER2 Protein are above the detection limit. The measured data of the samples S#2, S#3 and S#4 are statistically significant from zero, as tested with a two-sample t-test (p-values: S#2: 0.0001, S#3: 0.0012, S#4: 0.0046). In the case of S#5 (240,000-fold diluted sample), the sample input is below the detection limit (p-value: 0.1718) and will be excluded from the following RQ analysis.

20. In the case of the PICO Protein Detection Trial Kit, the applied dilution series of the sample and the known concentration of the recombinant human ErbB2/HER2 protein stock concentration (0.529 $\mu\text{mol/l}$) offers the opportunity to evaluate the sensitivity of the PICO assay in this experimental setup. The values of the 24,000-fold diluted sample (S#4) are within the measurable range of the assay. Based on the setup of the binding reaction (2 μl of sample is combined with 2 μl of the ABX), the concentration of HER2 protein in the binding reaction is 48,000-fold diluted from the stock concentration, resulting in a concentration of 1.10×10^{-11} M (equal to 11 pM).

Note that the theoretical limit of detection (based on analytical PICO models) is 4 complexes and the theoretical limit of quantification is 10 complexes. Due to the standard deviation, the experimental LOD is higher as demonstrated in this exemplary experiment. Based on this example, the average raw complex count of S#4, with the lowest measurable protein concentration, the average complex count is 12. Thus, one-twelfth of the complex count measured in S#4 could theoretically be measured and quantified leading to a theoretical LOQ of 0.9 pM and one thirtieth of the complex count could theoretically be measured, leading to a theoretical LOD of 0.37 pM.



21. The lambda-normalized and ABC-corrected complex counts of the replicates (ξ_A) from the samples within the detectable range are normalized to the amount of recombinant human ErbB2/HER2 protein in the binding reaction (ξ_T). This is necessary to compare samples with different amounts of input.

Note that depending on the sample type, the input in the binding reaction can be a measure of target (in this case the amount or concentration of a recombinant protein) or a cell count in the binding reaction.

$$\xi_T = \frac{\xi_A}{T}$$

ξ_T : ABC corrected and lambda normalized complex count
of one replicate normalized to input of the binding reaction

ξ_A : ABC corrected and lambda normalized complex count of the
replicate

T : Amount of input to the binding reaction

Example: The amount of recombinant human ErbB2/HER2 protein in the binding reaction of S#2, S#3 and S#4 is calculated based on the dilution series. The concentration of undiluted protein is 0.529 $\mu\text{mol/l}$. According to 09.3 step 5, each binding reaction contains 2 μl of protein dilution. For S#2 a 6,000-fold dilution, for S#3 a 12,000-fold dilution and for S#4 a 24,000-fold dilution of the protein stock was performed. Thus, each binding reaction sample of S#2 contains 88.2 pmol, S#3 44.1 pmol and S#4 22 pmol. Exemplarily the ξ_A of the sample in well A2 belonging to S#2 is 510.18 resulting in a ξ_T of 5,785.48.

22. To evaluate if two data sets which are compared have statistically significant different expression of the target, the ξ_T values of both samples are tested for statistically significant differences. Choose an appropriate statistical test depending on your experimental setup. Based on the theoretical background of PICO, a normal distribution of the data can be assumed.

Example: Due to the experimental setup of the PICO Protein Detection Trial Kit, ξ_T values of the samples S#2, S#3 and S#4, all differently diluted samples from the same recombinant ErbB2/HER2 protein stock are compared. Thus, a paired t-test can be used. Further, we do not observe statistically significant differences between all samples (p-values: S#2/S#3: 0.424, S#2/S#4: 0.137, S#3/S#4: 0.378) as expected, because the data was already compensated for the different amount of input. (The ξ_T values for all samples can be calculated based on the ξ_A values given in the example of step 21 and the sample input given in example of step 23.)



23. Next, the fold change between two samples is calculated using the average ξ_T values of two samples.

$$FC = \frac{avg(\xi_T (sample\ 1))}{avg(\xi_T (group\ 2))}$$

FC : Fold change of the ξ_T between both samples

avg(ξ_T (sample 1/sample 2) : Average lambda normalized, ABC

corrected and input normalized complex counts of sample 1 or 2

Example: The fold change using the average ξ_T values of S#2 (5,836.11), S#3 (6,503.00) and S#4 (7,560.00) samples were calculated resulting in $FC_{S\#2/S\#3}$ of 0.897, $FC_{S\#2/S\#4}$ of 0.772, and $FC_{S\#3/S\#4}$ of 0.860.

24. Transform the fold change values into log2 fold change values (results in $\log_2(FC)$). This value indicates the relative difference of the analyzed target present in the different samples (meaning the differences in the initial undiluted sample, not the diluted sample used for RQ).

Example: The fold change values are transformed into log2 fold change values ($\log_2(FC)_{S\#2/S\#3}$: -0.156, $\log_2(FC)_{S\#2/S\#4}$: -0.373, $\log_2(FC)_{S\#3/S\#4}$: -0.217). As expected, the values are close to zero, indicating no different expression between the samples.

25. The p-value can be transformed using $-\log_{10}$ (results in $-\log_{10}(p\text{-value})$). The log2 fold change can be plotted against the transformed p-value in a volcano plot.

When RQ is performed to compare the amount of target in two samples, we recommend to average the log2FC values and the p-values.

Example: The p-values ((p-values: S#2/S#3: 0.4241, S#2/S#4: 0.1369, S#3/S#4: 0.3778)) are transformed into $-\log_{10}$ values (S#2/S#3: 0.3725, S#2/S#4: 0.8635, S#3/S#4: 0.4227). The average log2 fold change is -0.249 and the average $-\log_{10}$ p-values is 0.553. Thus, in case of the experimental setup of the PICO Protein Detection Trial Kit, the relative differences between S#2, S#3 and S#4, all differently diluted samples from the same recombinant ErbB2/HER2 protein stock, result, as expected, in no significant difference in protein amount.



12 Troubleshooting Guide

Consult the troubleshooting guide below to solve any problems that may arise. For more information, see the [Frequently Asked Questions](https://www.actome.de/resources/downloads) (FAQ). It is available at: www.actome.de/resources/downloads

Reach out to the Actome Customer Service (info@actome.de) if you have any questions about the protocol.

Troubleshooting	
Issue	Comments and Suggestions
No complexes or low numbers of complexes detected	
Wrong default threshold of fluorescence intensity (RFU) was set in the QIAcuity software suite	Select '1D Scatter Plot' in analysis mode of QIAcuity software suite and adapt the thresholds.
Error from AMULATOR	
Single wells of the nanoplates are empty	Delete the corresponding wells in the sample definition file as well as the according table line to create a continuous table.



13 Ordering Information

PICO kits can be purchased directly from shop.actome.de or a quote can be requested from sales@actome.de. Supporting materials are available on www.actome.de/resources/downloads or can be requested from Actome's Customer Support (info@actome.de).

Ordering		
Product	Description	Cat.#
This Product		
PICO Protein Detection Trial Kit	Trial kit for the detection of recombinant HER2 using trastuzumab and pertuzumab	PICO-000090
Related PICO Products		
PICO Amplification Core Kit	dPCR detection for PICO assays (5 x 24 rxns)	PICO-000010
PICO Probes	BL (HEX), P8 (FAM), N6 (TAMRA), O7 (ROX) probes for detection in dPCR (5 x 24 rxns)	PICO-000070 - 73

