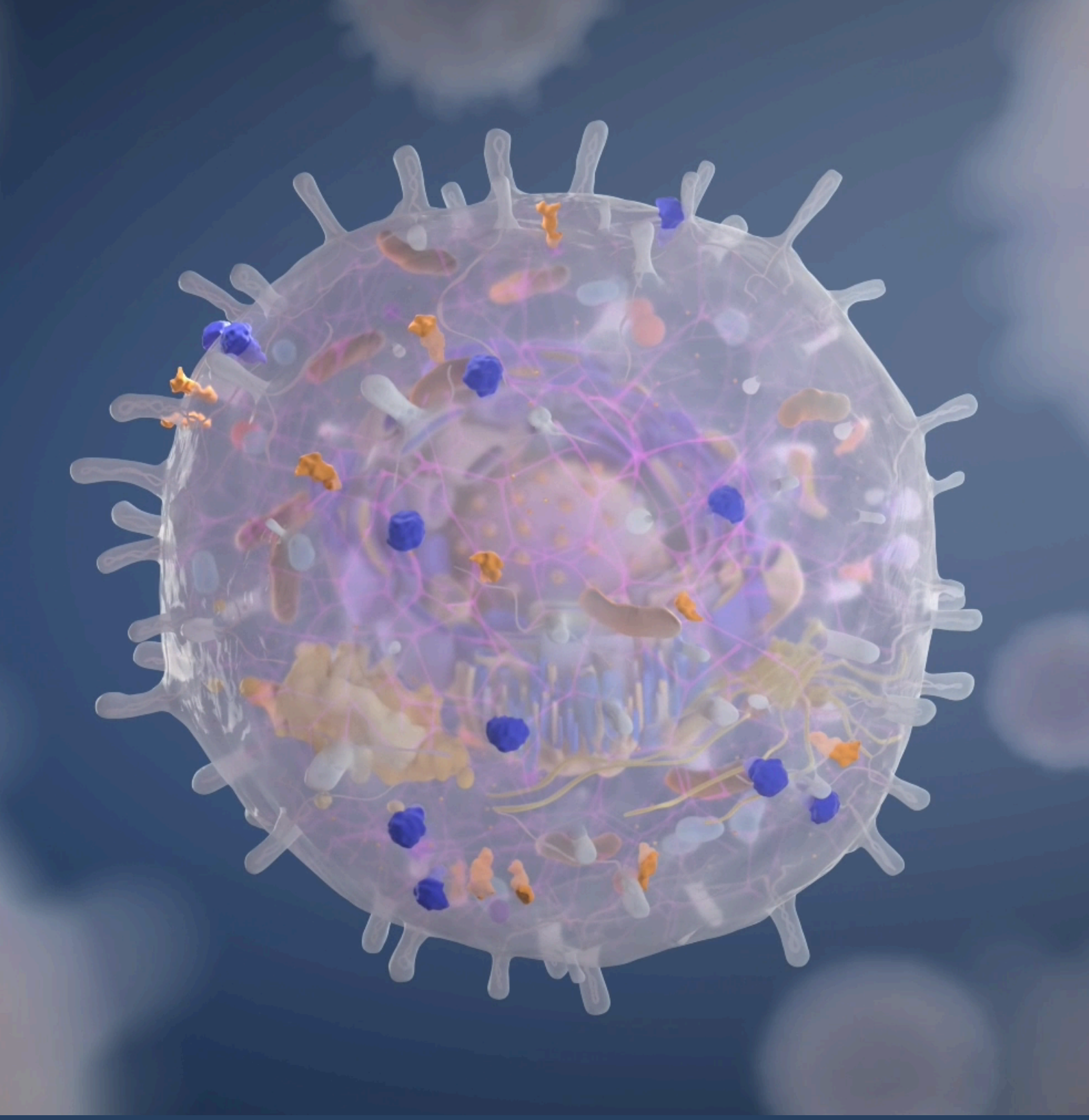

PICO Amplification Core Kit
USER MANUAL

actome

Actome GmbH
Georges-Köhler-Allee 302
79110 Freiburg
Germany

01 Kit Contents and Storage

PICO Amplification Core Kit		
Catalog Number:		PICO-000010
Pouch - store at 4°C	**pcs**	**Item code**
Additive T	1	A
Additive C	5	B
Bovine serum albumin	5	C
Pouch - store at -20°C		
Additive L	5	D
Coupling dPCR Mix	1	E

The PICO Amplification Core 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 Amplification Core Kit. Date of expiry is stated on the back side of the pouches.

02 Intended Use

The PICO Amplification Core 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 Amplification Core 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 the QIAcuity Digital PCR System. The PICO Amplification Core (AMC) Kit is compatible with both 24- and 96-well QIAcuity Nanoplates (26k and 8.5k respectively). It provides enough reagents for either five 24-well QIAcuity Nanoplates, enabling 120 PICO reactions, or five 96-well QIAcuity Nanoplates allowing the analysis of 480 individual PICO reactions. The corresponding PICO BL, P8, N6, or O7 Probe are required additionally dependent on which PICO Labels were used during the antibody labeling. The PICO AMC protocol can be completed with minimum hands-on time (< 1 h 30 min) as a two-day procedure (**Figure 1**).

The PICO assays can be performed using two, three, or four different labeled antibodies, dependent 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.

Two different evaluation options are available for PICO: **1)** relative quantification (RQ), using an external standard or control sample for internal reference, or **2)** absolute quantification (AQ), analysis without an external standard. To learn more about the different PICO quantification methods check out our PICO Quantification application note.

For the PICO assay the antibodies of your choice have to be labeled with PICO Labels (available in four versions: BL, P8, N6, or O7) using the PICOglue Antibody Labeling (gAL) Kit.

Day	Steps	Timing	Hands-on	Stopping points & Storage
Day 1	**Step 1 – Antibody Labeling**	**5 h 50 min**	**2 h 30 min**	
	A. Rebuffering of the Antibody	15 min	15 min	
	B. Deglycosylation of the Antibody	1 h 10 min	10 min	
	C. Azide Attachment	10 min + ON	10 min	overnight incubation (ON) at 30°C
Day 2	D. Cleaning up the Antibody	15 min	15 min	
	E. Label Attachment	10 min + ON	10 min	overnight incubation (ON) at 4°C
Day 3	F. Bioanalyzer - Labeling Efficiency	1 h 30 min	40 min	optional
	G. Removal of Free Labels	2 h 5 min	35 min	
	H. Rebuffering of the Antibody	15 min	15 min	stable for 12+ months at 4°C
	Step 2 – Antibody Quality Control	**2 h 40 min**	**25 min**	
	A. QIAcuity dPCR run	2 h 30 min	15 min	
	B. Calculation of Antibody Concentration	10 min	10 min	
Day 4	**Step 3 – Preparation of PICO Assay**	**approx. 20 min**	**approx. 20 min**	
	A. Preparation of Biological Material	sample dependent		
	B. Setup of Antibody Mix and Sample	20 min	20 min	
	Step 4 – PICO Assay	**3 h 20 min**	**1 h**	
	A. Binding Reaction	30 min + ON	30 min	overnight incubation (ON) at 4°C
Day 5	B. Dilution of the Samples before dPCR	20 min	20 min	
	C. QIAcuity dPCR Run	2 h 30 min	10 min	overnight run possible
	Step 5 – Evaluation			

Figure 1. The PICO Amplification Core Kit workflow

06 Preparation for a PICO Assay

06.1 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.

06.2 Concentration of Antibodies in the Binding Reaction

PICO assays require at least two antibodies per target, which should bind to two different, concurrently accessible epitopes. Requirements and suggestions regarding antibody selection can be found in the PICOglue Antibody Labeling Kit User Manual.

- If absolute quantification is ***not*** required we recommend using an antibody concentration of 4×10^{-11} M in the binding reaction (see section *08.3 Binding Reaction)*.
- **Important:** For absolute quantification, the assay has to be conducted in saturated condition. In this case, we recommend an antibody concentration of 5×10^{-10} M in the binding reaction.
    - Please note that increasing the antibody concentration can reduce the sensitivity of the assay.
- For optimizing the antibody concentration, an isomolar titration (IMT) of the antibody concentration can be performed. For this, we recommend an antibody concentration range between 1×10^{-9} M and 1×10^{-13} M in the binding reaction.
    - Please contact us for more information on how to perform an IMT experiment.

06.3 Considerations Regarding the Biological Material

Biological Material		
Feature	Main criteria	Additional criteria
Sample type	Any solubilized biological sample	Different sample preparation steps for homogenization is necessary
Sample preparation	Sample dependent	• Lyse cultured/primary cells using Actome's lysis buffer (LB) • Mechanical/enzymatical homogenization of tissue material
Buffer	Lysis buffer (LB)	Actome's lysis buffer (LB) is suitable for most biological materials, however in some cases adaptation might be necessary
Homogenization	Sonification and QIAShredder Spin Column treatment (Cat.#: 79656)	
Stabilization of proteins/ interactions	optional	• Use 1x PIC-PBS for all washing steps during cell preparation (cOmplete Protease™ Inhibitor Cocktail (Roche, Cat.#: 04693132001))
Stability of prepared sample	Sample dependent	Cell lysate can be stored at 4°C for up to 4 days, however we recommend using it immediately. Freezing of cell lysate is also an option (see *Section 08.2*)

- As an input for cell lysis, we recommend using a total of 1 million cells in a concentration of 1×10^4 cells/µl. However, total input and cell concentration can be adapted to the user's needs.
- For optimization, the input of biological material in the binding reaction can be titrated using a constant antibody concentration (CLC) experiment. The dilution generating the highest couplex numbers can then be used as reference for other experiments.
- For high expressing targets, we recommend using 3-fold dilution series. Thus, performing a total of five dilution steps results in a dilution series of undiluted, 3-, 9-, 27- and 81-fold diluted samples.
- For low expressing targets, we recommend using 2-fold dilution series. Thus, performing a total of five dilution steps results in a dilution series of undiluted, 2-, 4-, 8- and 16-fold diluted samples.

06.4 Exemplary Setup of a Binding Reaction for a 24-well dPCR Nanoplate

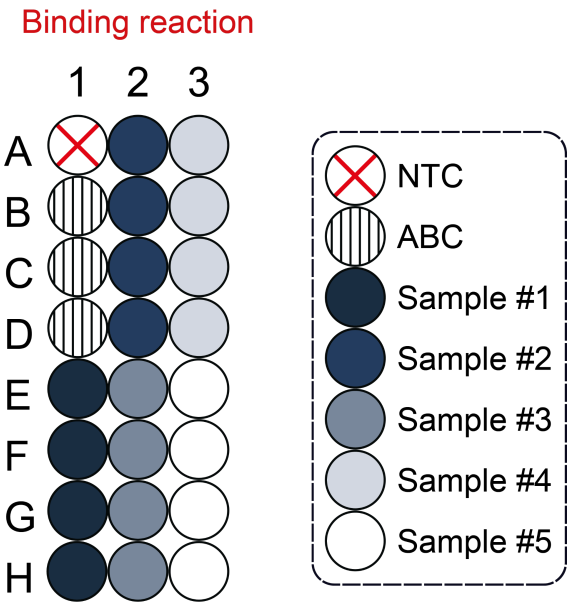

Figure 2. Plate layout of an example binding reaction for a 24-well PICO experiment. In this example five samples, with four technical replicates each, were used. For the ABC three technical replicates are sufficient. ABC - antibody control; NTC - non template control

06.5 Exemplary Setup for dPCR Pre-dilution

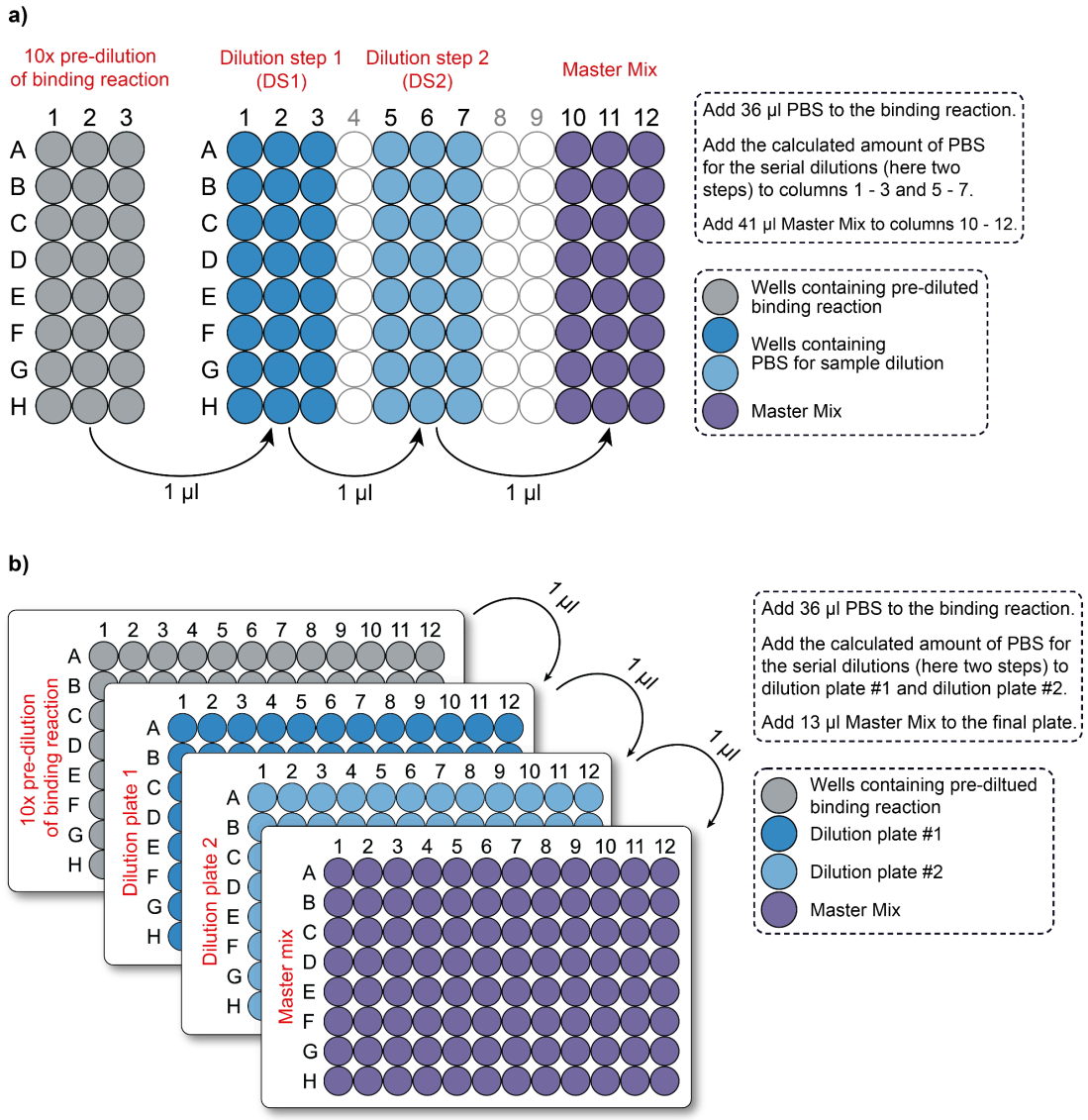

Figure 3. Exemplary setup to perform the dPCR pre-dilution in **a)** 24- or **b**) 96-well QIAcuity Nanoplates. The '10x pre-dilution of binding reaction' is prepared in the plate containing the binding reaction.

The setup of a two-step dPCR pre-dilution in a 24- or 96-well QIAcuity Nanoplate can be seen in **Figure 3**. For a 24-well PICO assay a 96-well plate is prepared with columns 1 - 3 and 5 - 7 containing the calculated amount of PBS. In columns 10 - 12 41 µl of Master Mix is added (**Figure 3a**). For a 96-well PICO assay three additional 96-well plates are prepared. Two of them (Dilution plate #1 and #2) are filled with the calculated amount of PBS while the third plate is filled with 13 µl of Master Mix (**Figure 3b**). 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) (**Figure 3a**) or the Dilution plate #1 (**Figure 3b**) and the dilution is mixed by pipetting 30 times. The dilution is repeated in Dilution step 2 (DS2) and Dilution plate #2. Finally, 1 µl of DS2/Dilution plate #2 is transferred to the wells/plate containing the Master Mix. After mixing by pipetting three times, 40 µl/12 µl of the Master Mix, containing the diluted sample, are transferred to a 24- or 96-well QIAcuity Nanoplate respectively and dPCR is performed. Perform the dilution steps as fast as possible since the dilution disrupts the equilibrium binding conditions and induces antibody dissociation.

07 Equipment and Reagents to be Supplied by User

Notes

Note: Equipment and reagents necessary for preparation of biological material is not listed below and are dependent on the input material.

Devices/Equipments

- 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)
- Multichannel pipette, 8-channel (30 – 300 µl) (optional)
- 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) or QIAcuity Nanoplate 8.5k 96-well (QIAGEN, Cat.#: 250021)
- 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
- 0.5 ml low protein binding tubes (e.g. Eppendorf, Cat.#: 0030108094)
- 10 µl, 200 µl, 1,000 µl standard pipette tips
- 15 ml falcon tubes

Chemicals and Kits

- QIAcuity Probe PCR Kit (QIAGEN, Cat.#: 250101) (~ 1.5 ml)
- PICO Probes (PICO BL, P8, N6, O7 Probe, Cat.#: PICO-000070-73)
- 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)

Labeled Antibodies

- Up to four labeled antibodies prepared by the PICO gAL Kit (Cat.#: PICO-000110) using PICOglue BL, P8, N6, or O7 Labels (Cat.#: PICO-000120-123)

08 Protocol of PICO Amplification Core Kit

Note: The protocol is valid for PICO assays using up to four different antibodies labeled with the PICOglue BL, P8, N6, or O7 Labels. The protocol explains the setup of a QIAcuity Nanoplate 26k 24-well or a QIAcuity Nanoplate 8.5k 96-well. Both plate types can be used for PICO assays. Please consider that a lower number of partitions per sample (as in the QIAcuity Nanoplate 8.5k 96-well) reduces the sensitivity of the assay.

08.1 Buffer Preparation

1. Prepare the chemicals and buffers as listed below. The volumes can be adapted to the needs (e.g. if required more LB can be prepared for the dilution series).
   The buffers can be kept for the specified periods of time and used for further experiments. Prepare the buffers directly when they are used.

Additive C (5x stock)
Add 500 µl PBS
Easily soluble, stable for 1 week at 4°C

BSA (5x stock)
Add 400 µl PBS
Stable for 3 days at 4°C

EDTA-free Protease Inhibitor Cocktail (PIC), (25x stock)
1 tablet of cOmplete Protease™ Inhibitor Cocktail
2 ml PBS
Stable for 12 weeks at -20°C

Lysis Buffer Stock (LB-Stock), (2x stock)
200 µl Additive T
400 µl Additive C
80 µl PIC
200 µl Additive L
120 µl PBS
Stable for 3 days at 4°C

Lysis Buffer (LB)
300 µl LB-Stock
300 µl PBS
Stable for 3 days at 4°C

Control Buffer (CB)
250 µl LB-Stock
100 µl BSA
150 µl PBS
Prepare the Control Buffer fresh

PICOglue Storage Buffer
20 µl 10x PICOglue Antibody Storage Buffer - from PICO gAL Kit
180 µl PBS
Stable for 6 months at 4°C

Buffers optional for Preparation of Biological Material

PIC-PBS (1x)
1 PIC tablet
50 ml PBS
Stable for 2 weeks at 4 to 8°C
Stable for 12 weeks at -15 to -25°C

BS3 Stock Solution (BS3S) 100 mM (20x stock)
2 mg BS3
37.8 µl DMSO
Stable at -20°C for several months

BS3 Working Solution (BS3W) 5 mM
6 µl BS3S
114 µl PBS
Prepare the BS3W fresh

08.2 Preparation of Biological Material - Exemplary Procedure For Cultivated Cells

Note: Adaptations and changes are necessary depending on the biological material used. For recommendations see *06.3 Requirements for Biological Samples*.

2. Prepare PIC-PBS solution and LB according to *step 1*. If cross-linking is desired, we recommend using 5mM BS3 (e.g. BS3, Thermo Fisher Scientific, Cat.#: A39266).
   The advantages or disadvantages of cross-linking must be discussed individually for each setup. We particularly recommend cross-linking when analyzing protein interactions. Please note that BS3 is only suitable for cell surface membrane proteins.

3. Remove the medium from the flask, add PIC-PBS to wash the cells carefully and remove the PIC-PBS.
   The amount of PIC-PBS depends on the flask size. We recommend using between 3 ml and 15 ml.

4. Harvest the cells using a cell scraper (e.g. Sigma Aldrich, Cat.#: C5981-100EA).

5. Add PIC-PBS and transfer the cells to a 15 ml falcon. Centrifuge (400 rcf, 5 min) and discard the supernatant.
   The amount of PIC-PBS depends on the flask size. We recommend using between 3 ml and 15 ml.

6. Resuspend the cells in 1 ml of PIC-PBS, transfer to a 1.5 ml reaction tube, centrifuge (400 rcf, 5 min) and discard the supernatant. Repeat the washing with 1 ml of PIC-PBS, centrifuge (400 rcf, 5 min) and discard the supernatant. If cross-linking is required continue with step 7 otherwise skip ahead to step 10.
   If a cross-linker is used later, do not use PIC-PBS at this step, otherwise the PIC will quench the BS3 crosslinker.

7. Resuspend the cells in 1 ml of PBS. Count the cells and transfer 2 × 10^6 cells into a new 1.5 ml reaction tube, centrifuge (400 rcf, 5 min) and discard the supernatant.
   Consider the total cell count, not the viable cell count. Depending on the experimental setup and the desired input, the concentration of the cells can be adapted. If so, the amount of BS3W has to be adapted.

8. Crosslink the cells by resuspending them in 100 µl of BS3W. Mix thoroughly by pipetting up and down five times and incubate for 30 min at room temperature.
   Prepare BS3W fresh shortly before use.

9. Add 900 µl of PIC-PBS, centrifuge (400 rcf, 5 min) and discard the supernatant.

10. Resuspend the cells in 1 ml of PIC-PBS. Count the cells and transfer 1 × 10^6 cells into a new 1.5 ml reaction tube.
    Use the total cell count, not the viable cell count. In case you have less than 1 × 10^6 cells, reduce the LB volume in step 12 to have a cell concentration of 1 × 10^6 cells per 100 µl (=1 × 10^4 cells/µl). Depending on the experimental setup and the desired input, the concentration of the cells can be adapted.

11. Centrifuge (400 rcf, 5 min) and carefully discard the supernatant.
    Discard the supernatant carefully not to lose cells, as the number of the cells in the assay is defined at this step.

12. Resuspend the cells in 100 µl of LB and vortex for 10 s. Lyse the cells for 3 h at 4°C.
    Prepare the antibody mix (ABX) during the incubation time (see *08.3 Binding Reaction 16-19.*). Keep leftovers of the LB and LB-Stock for the following steps.

13. Sonicate the lysate for 5 min at full power in an ultrasonic bath at room temperature. Use ice if the temperature reaches critical level for your samples.

14. Transfer lysate into a QIAshredder spin column (QIAshredder, QIAGEN, Cat.#: 79656) and centrifuge at full speed (~20,000 rcf) for 2 min. Transfer the flow-through to a new 1.5 ml reaction tube. The lysate is ready to use.
    The lysate can be stored for 4 days at 4°C, however we recommend using it directly. Additionally, cell lysate can be aliquoted and frozen at -20°C for at least a month. However, changes in the assay readout due to the freezing process cannot be ruled out, thus for comparative assays we recommend treating and storing all samples in the same way.

08.3 Binding Reaction

15. Calculate the volume of antibody stocks, LB-Stock, and PBS required for the antibody mix (ABX) using the PICO Calculator (Step 2 in the 'PICO Calculator' tab). To calculate the ABX manually check *section 10.1 Calculation of the ABX Mix*.

    To avoid contamination, we recommend sterile handling of the antibody solutions. 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.

16. Prepare the ABX using LB-Stock. Then add the calculated volume of the antibody stock solutions or antibody pre-dilutions and fill up the remaining volume up with PBS.

    We recommend preparing a minimum of 60 µl of ABX for a QIAcuity Nanoplate 26k 24-well and 220 µl for a QIAcuity Nanoplate 8.5k 96-well. For relative quantification, we recommend using an antibody working concentration of 4×10^{-11} M and for absolute quantification 5×10^{-10} M. This can be selected from the drop-down menu in the PICO Calculator.

    Example: The total volume of the ABX is 100 µl. 50 µl of LB-Stock is added in a low protein binding tube. The volume of the first antibody stock added to the ABX is 22.41 µl. The volume of the second antibody stock added to the ABX is 9.27 µl. The volume of the third antibody stock added to the ABX is 2.42 µl. The volume of the fourth antibody stock added to the ABX is 4.25 µl. The volume remaining to fill up to 100 µl (11.65 µl) is filled up with PBS.

17. After preparation of the ABX, the biological material prepared in *section 08.2* can be diluted or used directly for the following binding reaction.

    To conduct a CLC experiment (constant antibody concentration) please refer to the recommendations in *Section 06.3*.

18. Set up the binding reaction in a 96-well PCR microplate (v-bottom) (see example in **Figure 2**). For the NTC add 4 µl of LB to a dedicated well.

19. For the ABC control, combine 2 µl of CB with 2 µl of ABX and make at least three technical replicates.

    Due to the small volume of the binding reaction, do not mix by pipetting. The volume of lysate and ABX can be adapted. This must be considered in the calculations of the ABX and the pre-dilution. Please keep in mind that multiple different ABC control samples might be necessary, if different antibody mixes are used in one experimental setup.

20. For the biological samples combine 2 µl of sample with 2 µl of ABX. As a good laboratory practice, it is advisable to use at least four replicates.

21. 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.

08.4 Pre-dilution and Digital PCR

22. Prepare the Master Mix for 24 or 96 samples. Make sure that the Master Mix contains the PICO Probe(s) needed. If less than four different labeled antibodies are used, replace the volume of the PICO Probe(s) with ultrapure water (see amounts below). Vortex for 10 s and spin down (~1,000 rcf, 5 s).
    Store the Master Mix at 4°C until used. The Master Mix can be prepared up to three days in advance.

Master Mix		
Reagents	**24-well plate**	**96-well plate**
Ultrapure water	606 µl	834 µl
QIAcuity Probe Master Mix	284 µl	390 µl
PICO Probe (P8, BL, N6, or O7)	45 µl	62 µl
PICO Probe (P8, BL, N6, or O7)	45 µl	62 µl
PICO Probe (P8, BL, N6, or O7)	45 µl	62 µl
PICO Probe (P8, BL, N6, or O7)	45 µl	62 µl
Coupling dPCR Mix	36 µl	50 µl

23. Prior to dPCR, the samples have to be diluted. We recommend aiming for a lambda (average number of targets per partition) of 0.15, however a range between 0.01 - 0.6 is acceptable.
    The sensitivity of the assay is 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.

24. 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). Make sure to choose the correct Nanoplate in the PICO Calculator. To calculate the dilution series manually follow the steps in *section 10.2 Calculation of the Dilution Factor.*

25. For a 24-well PICO assay prepare a new 96-well plate for the dilution steps. Add the calculated amount of PBS to columns 1 - 3 and 5 - 7 and 41 µl of Master Mix to columns 10 - 12. For a 96-well PICO assay prepare three 96-well plates. Add the calculated amount of PBS to Dilution plate #1 and #2 and add 13 µl Master Mix to the third 96-well plate (see example setup for 24- or 96-well QIAcuity Nanoplates in **Figure 3**).

26. Remove the adhesive foil from the incubated sample plate carefully. Add 36 µl of PBS to the 96-well plate containing the binding reactions

(represents the recommended 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.

27. Transfer the predetermined carry-over volume from each pre-diluted sample into the corresponding wells of the dilution plate (DS1/Dilution plate #1) (see **Figure 3**). Mix by pipetting up and down 30 times.

28. When performing the second dilution step, transfer the predetermined carry-over volume from the first dilution step into the corresponding wells of the dilution plate (DS2/Dilution plate #2) (see **Figure 3**). Mix by pipetting up and down 30 times.

29. Finally, transfer 1 µl from the last dilution step into the wells containing the Master Mix . Mix by pipetting up and down 30 times.

30. Transfer 40 µl or 12 µl of the Master Mix, containing the diluted samples, to a 24- or 96-well QIAcuity Nanoplate respectively. Seal the Nanoplate according to the QIAcuity user manual 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**	
Hot-start	95°C	2 min	
Denaturing	95°C	15 s	Cycle 40 times
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
N6 Probe	TAMRA, orange channel	400 ms	6
O7 Probe	ROX, red channel	300 ms	4

09 Evaluation

09.1 Raw Couplex Calculation using AMULATOR

31. After the dPCR run, select 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’.

32. 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.

33. 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.

34. 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.

35. 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).

36. The AMULATOR can be accessed here: https://amulator.actome.de/

37. 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.

38. 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).

39. Select the project you would like to analyze and click on ‘Calculate Results’ to initiate the data analysis.

40. AMULATOR creates two graphs in the Results tab. The couplex chart displays the number of couplexes 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.

41. To download the results click on the ‘Export data’ button. To download the graphs click on the 'Export graph images’.

09.2 Absolute Quantification

Note: Absolute Quantification can only be performed if the labeling efficiency of the used antibodies is known, saturated conditions in the binding reaction have been used (see *Section 06.2*), and the samples are on the low antigen side. For reference please read our app note on PICO Quantification.

42. The raw couplexes from the AMULATOR are used as an input for AQ. Download the raw couplex count **without ABC correction** from the AMULATOR software (see *09.1 step 39*).

43. Transfer the raw couplex counts into the corresponding cells of the ‘AQ Evaluation Data’ tab in the PICO Calculator.

44. The molar concentrations and mean and median values are calculated automatically.

10 Appendix - Calculations

10.1 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* $[cp/\mu l]$
This concentration is determined via dPCR in the ABQC (see gAL Kit user manual)
N_A : *Avogadro constant* $(6.02214076 \times 10^{23}\ mol^{-1})$

Example: The concentration of an antibody stock, determined via dPCR (C_C) is 2.15×10^8 cp/µl. The molar concentration is calculated (3.57×10^{-10} M).

2. Calculate the volume of each antibody (V_{AB1}, V_{AB2}, V_{AB3} or V_{AB4}) used in the ABX based on the following calculation.
   If $V_{AB1\text{-}4}$ are too low for precise pipetting, a pre-dilution of the antibody stock in 1x PICOglue Storage Buffer can be made in low protein binding tubes. 10x PICOglue Storage Buffer from the PICO gAL Kit can be used and diluted in PBS. In this case, the concentration of the pre-dilution of the antibody (DF_{AB}) has to be considered in the calculation. Please note that highly diluted antibodies are shorter lasting.

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

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 4×10^{-11} *M* for relative quantification
and 5×10^{-10} *M for absolute quantification*

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 3.57×10^{-10}. We choose 100 µl as the total volume of the ABX and a concentration of 4×10^{-11} M of the antibody 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 volume of antibody stock added to the ABX is 22.41 µl.

10.2 Calculation of the Dilution Factor

1. 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* [$cp/\mu l$]

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

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

Example: The concentration of antibodies in the binding reaction is 4×10^{-11} M. The concentration in cp/µl is 2.4×10^7.

2. Calculate the targeted antibody concentration for the dPCR.

$$C_d = \lambda/V_p$$

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

λ: *Average number of targets per partition; we recommend* 0.15 (*range*: 0.01 - 0.6)

V_p: *Volume of a partition* [μl]; 0.00078 µl (*or* 0.00034 µl*l*

0.00078 µl *for QIAcuity Nanoplate* $26k$ 24 *well or*
0.00034 µl *for QIAcuity Nanoplate* $8.5k$ 96 *well*

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 cp/µl.

3. 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. This is 42 for if using a QIAcuity Nanoplate 26k 24-well or 13 if using a QIAcuity Nanoplate 8.5k 96-well.

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

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 *or* 13

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.

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

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

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

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 Troubleshooting Guide

Consult the troubleshooting guide below to solve any problems that may arise. For more information, see the Frequently Asked Questions (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
Lambda value in PICO assay not in range (0.01-0.6)	
Antibody concentration determined during quality control of labeled antibodies was not correct	Recalculate antibody concentrations using the data of the PICO assay and repeat the assay with the new concentrations. For this, the antibody concentration of each antibody found in the 'Current Results' file of the PICO assay is multiplied with the dilution factor back to the antibody stock for the corresponding antibody (DF_{BS}). The dilution factor back to the antibody stock concentration can also be calculated using the PICO Calculator.
No couplexes or low numbers of couplexes detected	
Antibody concentration determined during quality control of label loaded antibodies was not correct	Check if lambda value is in range (0.01-0.6), if not recalculate concentrations based on the data of the PICO assay and repeat the assay with the new concentrations.
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 PICO 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.

12 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 PICO Product		
PICO Amplification Core Kit	dPCR detection for PICO assays (5 x 24 rxns; 5 x 96 rxns)	PICO-000010
Related PICO Products		
PICO Probes	BL (HEX), P8 (FAM), N6 (TAMRA), O7 (ROX) probes for detection in dPCR (5 x 24 rxns)	PICO-000070 - 73