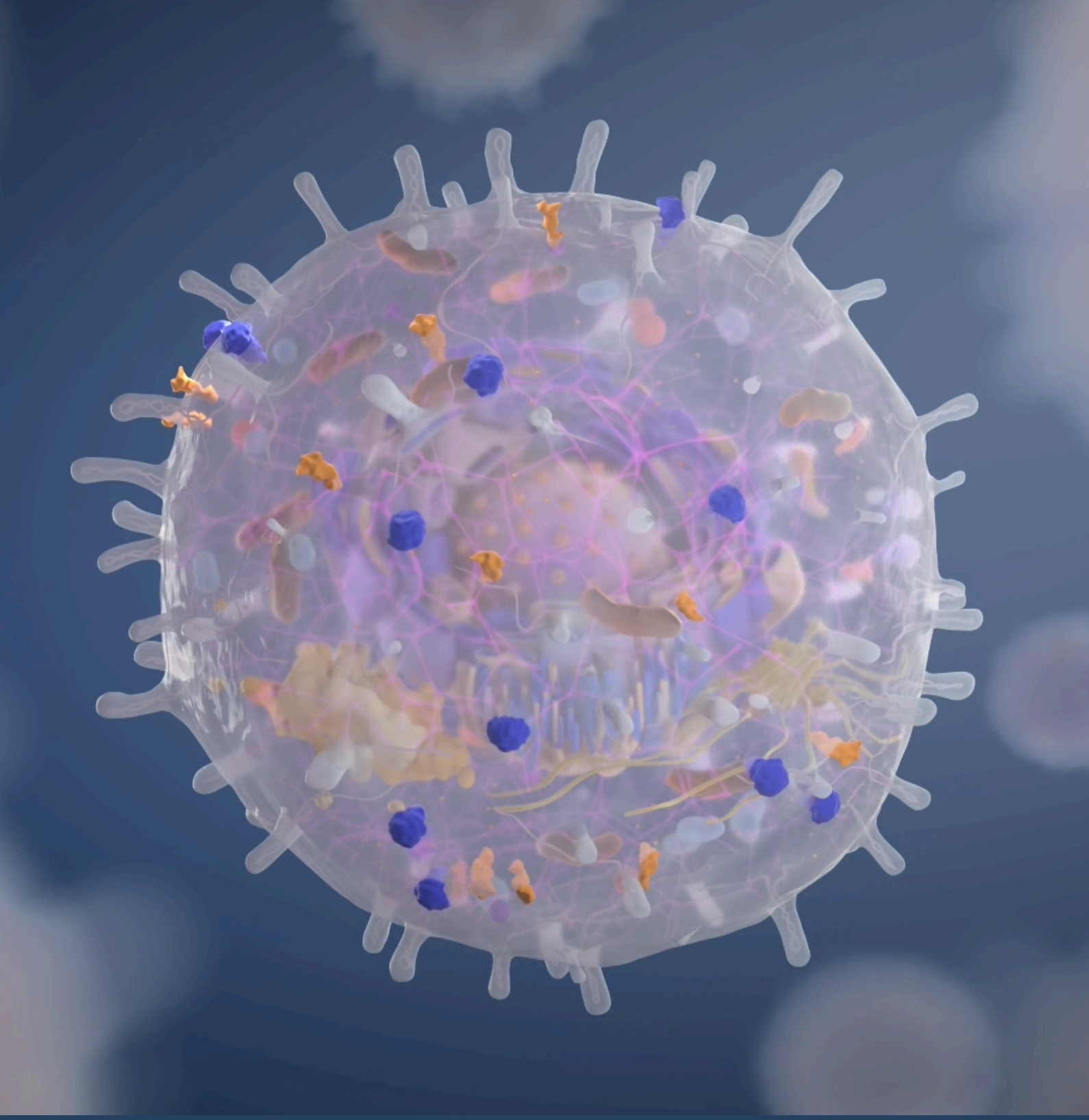


PICOglue Antibody Labeling Kit
USER MANUAL

actome

Actome GmbH
Georges-Köhler-Allee 302
79110 Freiburg
Germany

01 Kit Contents and Storage

PICOglue Antibody Labeling Kit			
Catalog Number:			PICO-000110
Pouch - store at 4°C	**pcs**	**Item code**	**Color code**
10x Ultrafiltration Buffer	1	A	
PICOzyme	1	B	
10x PICOtransferase Buffer A	1	C	
10x PICOtransferase Buffer B	1	D	
PICOtransferase	1	E	
PICOglue Antibody Binding Resin	4	F	
PICOglue Elution Buffer	1	G	
Pouch - store at -20°C			
10x PICOzyme Buffer	1	H	
PICOtransferase Substrate	4	I	
10x PICOglue Antibody Storage Buffer	1	J	
Pouch - store at RT			
2x Wash Buffer I	1	K	
2x Wash Buffer II	1	L	
100K Ultrafiltration Column	16	M	
2 ml Collection Tube	32	N	
Loading Column	4	O	

The PICOglue Antibody Labeling 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 PICOglue Antibody Labeling Kit. Date of expiry is stated on the back side of the pouches.

02 Intended Use

The PICOglue Antibody Labeling 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 PICOglue Antibody Labeling Kit is tested against predetermined specifications to ensure consistent product quality.

The PICOglue Antibody Labeling Kit passes quality control if it meets the following parameters using our standard testing antibody (recombinant antibody, Amgen):

- Labeling efficiency > 70 %

05 Introduction

The PICOglue Antibody Labeling (gAL) Kit is designed to enable the labeling of antibodies with DNA oligonucleotides (PICO Labels). The kit contains sufficient reagents for four antibody labeling reactions. It offers exceptionally high labeling efficiency (up to 100%), enabling highly sensitive PICO measurements. The PICO gAL protocol is performed as a three-day procedure with minimum hands-on time (~ 2h 30 min) (**Figure 1**). Four different PICO Labels (PICOglue BL, P8, N6, and O7 Label) are available for the labeling process (has to be purchased separately). At least two labeled antibodies, directed against the same target (e.g a single protein, each partner of a protein interaction, or against a protein and its post-translational modification), are required for a PICO assay.

The labeled antibodies are stable for up to one year at 4°C. The quality control step, using the Bioanalyzer System (Agilent), is designed to assess the labeling efficiency of the labeled antibodies. In addition, the PICO assay requires precise determination of the labeled antibody concentration, which is done by dPCR using components of the PICO Amplification Core (AMC) Kit and QIAGEN consumables.

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 PICOglue Antibody Labeling Kit workflow.

06 Preparation for the PICOglue Antibody Labeling Kit

06.1 Antibody Requirements

PICO assays require at least two labeled antibodies per target, which must bind to two different, simultaneously accessible epitopes. Since there are no washing steps (homogeneous assay) and no solid phase or other sterically challenging binding conditions exist during the PICO assay, careful antibody selection is usually not required. However, if multiple options are available, we recommend selecting antibodies based on the properties listed in the table below.

Antibody selection

Feature	Main criteria	Additional selection criteria
Tested application	Any application	In PICO, the target proteins are not denatured and natively folded proteins are detected. Therefore, using antibodies tested for applications without denaturing conditions (e.g. flow cytometry) is advisable.
Host	Any host species	Selection of rabbit antibodies might increase the sensitivity of the PICO assay due to the typically lower dissociation constant (K_D) of rabbit antibodies.
Class	Monoclonal, polyclonal IgG	We recommend selecting monoclonal antibodies to improve binding specificity.
Epitope	Any epitope	Select antibodies targeting epitopes which are freely available and not masked by other antibodies or proteins, and ensure non-competitive and simultaneous binding.
Buffer composition	For optimal results, the buffer should **not** contain carrier proteins (e.g. BSA or gelatin).	If this is not possible, the labeling reaction will not be affected, however the evaluation of the labeling and deglycosylation efficiencies may be compromised because BSA can mask the heavy chain (see S*ection 12 Troubleshooting Guide)*.

06.2 Concentration of Antibodies

Use a total amount of 50-100 µg antibody for labeling with the PICOglue Antibody Labeling Kit. If the volume exceeds 400 µl, add it to the Ultrafiltration Column in multiple steps and concentrate the antibody. Always centrifuge at 14,000 rcf for 3 min at 4°C for each step and discard the flow-through. Repeat these steps until the total amount of antibody is collected. Continue with the protocol from *section 08.1 step 3*.

06.3 Handling of Ultrafiltration Columns

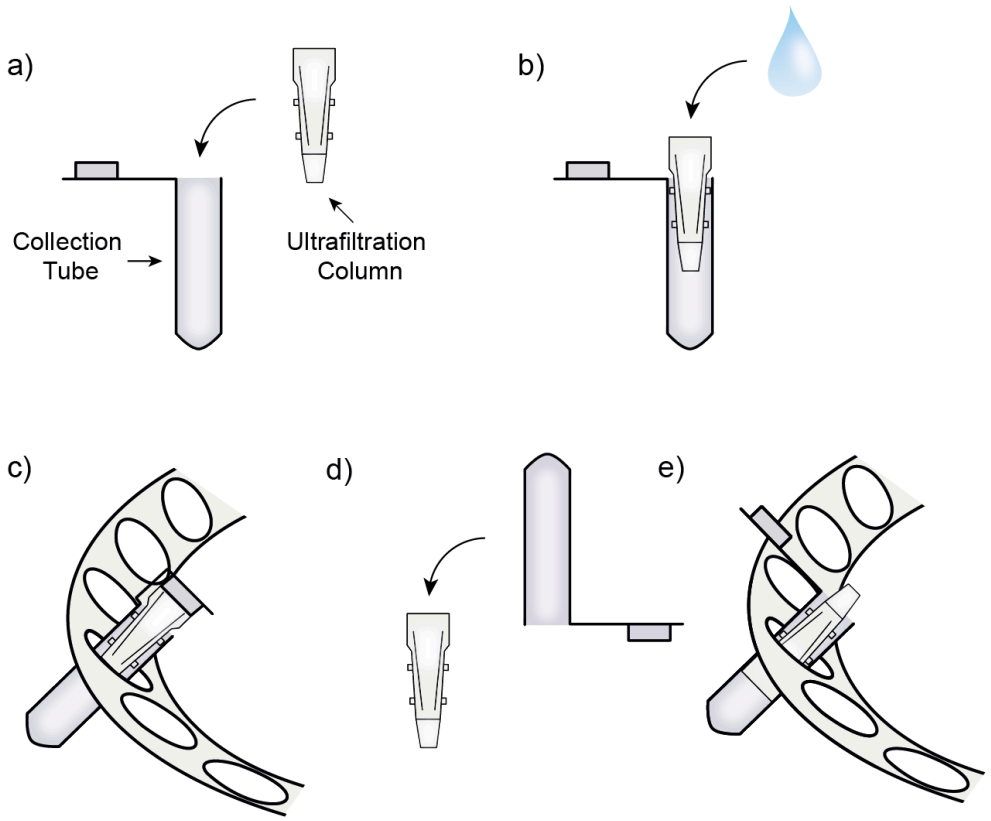


Figure 2. Ultrafiltration. (a) Place the Ultrafiltration Column into the Collection Tube, (b) load the liquid and cap the Ultrafiltration Column with the tube cap, (c) place the tube with the hinge of the cap and the side of the Ultrafiltration Column membrane outward, centrifuge it according to the protocol step. Usually 14,000 rcf 3-15 min is used depending on the membrane type and the desired concentration. Always follow the protocol. (d) To recover the sample, place a new Collection Tube over the Ultrafiltration Column invert it and, (e) spin (1,000 rcf, 20 s). The concentrated volume should be between 15-40 µl. Take care not to break off the lid of the Collection Tube and use closed centrifuge rotors.

06.4 Handling of Loading Columns

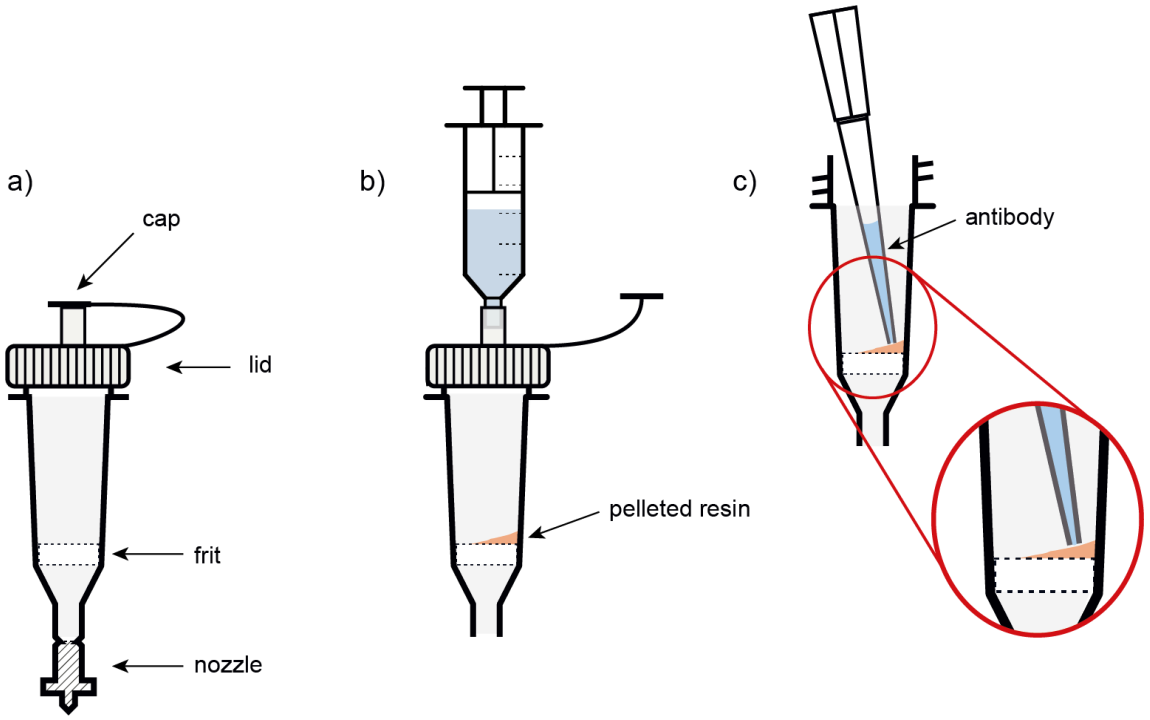


Figure 3. Applying the antibody into the Loading Column (*Section 08.6*). (a) Schematic depiction of the Loading Column. (b) After centrifugation, the resin forms a tilted surface. For washing, close the lid and open the cap. Attach the syringe to the opening and wash carefully with gentle but constant pressure. (c) For pipetting, unscrew the lid of the Loading Column and open it. The antibody solution should be dispensed onto the middle of the tilted resin surface directly. Do not touch the plastic walls or the frit. After pipetting, close the lid and incubate for 1 h.

06.5 Exemplary Setup for Quality Control dPCR of Two Labeled Antibodies

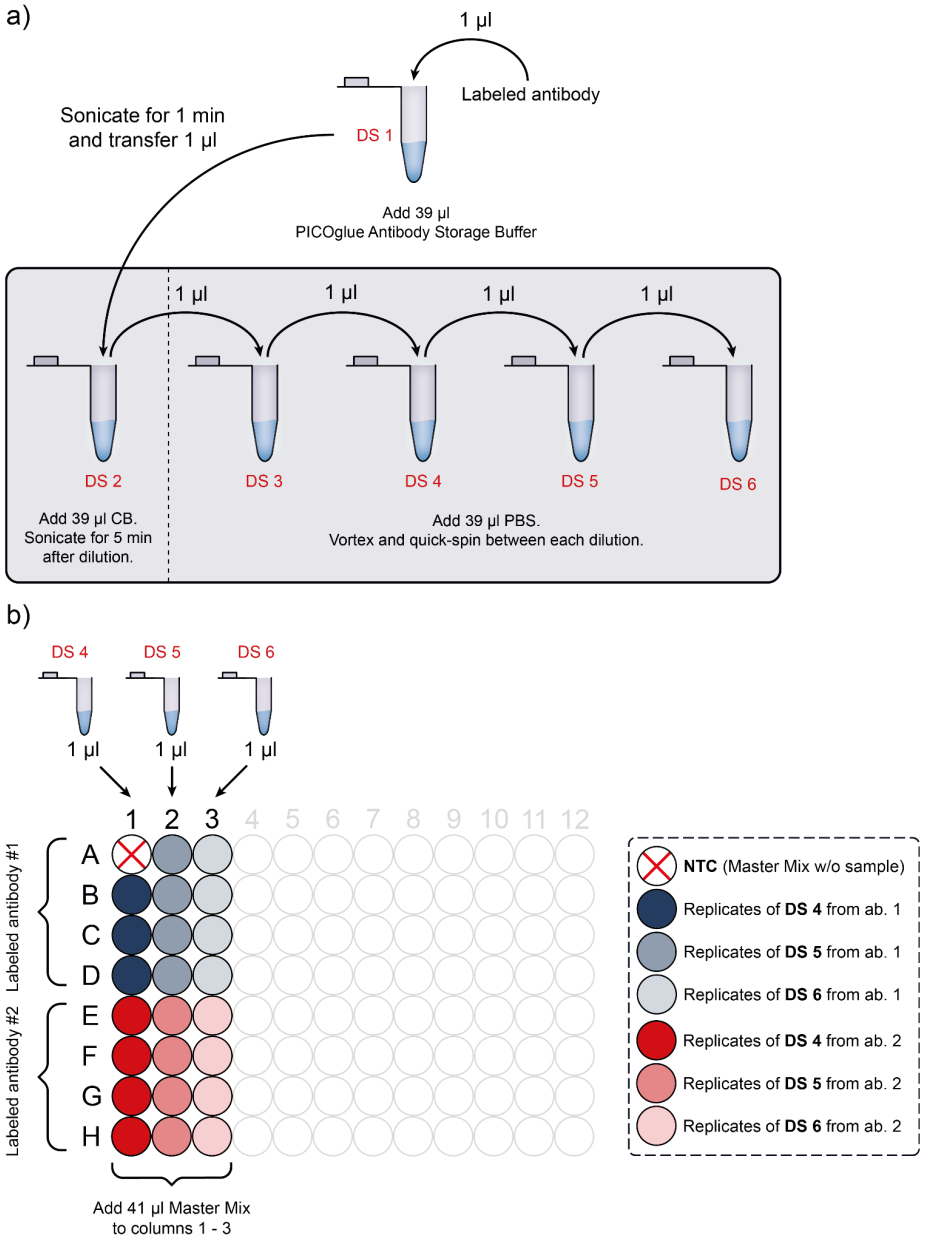


Figure 4. Exemplary setup to perform the Quality Control dPCR of two labeled antibodies. Here, four replicates of each labeled antibody are prepared. DS - dilution step; NTC - non template control

The setup of the Quality Control dPCR of two labeled antibodies can be seen in **Figure 4**. First, the antibody is diluted 1:40 in PICOglue Antibody Storage Buffer in low protein binding tubes (Dilution step 1 (DS1)) and sonicated for 1 min. Next the labeled antibody dilution (DS1) is diluted 1:40 in CB Buffer in a reaction tube (DS2) and sonicated for 5 min. The following 1:40 dilution steps (DS3-6) are performed serially in PBS using reaction tubes. Vortex and quick-spin between each dilution (**Figure 4a**). Next prepare a 96-well plate with 41 µl of Master Mix in columns 1 - 3. Transfer 1 µl of diluted antibody from the last three dilution steps (DS4-6) to the wells containing the Master Mix and mix by pipetting. Prepare four technical replicates. Please keep in mind that no sample should be transferred in well A1 for the non template control (NTC) (**Figure 4b**). After mixing by pipetting, transfer 40 µl of the Master Mix containing the diluted samples to a QIAcuity Nanoplate 26k 24-well for dPCR amplification.

07 Equipment and Reagents to be Supplied by User

Notes

Devices/Equipment

- QIAcuity Digital PCR System (QIAGEN, Cat.#: 911001)
- 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, 5-12.5µl - optional
- Refrigerated centrifuge for 1.5 – 2 ml reaction tubes
- Heating block for 1.5 ml reaction tubes or standard PCR machine
- Table-top mini centrifuge for quick spins (~1,000 rcf)
- Vortex mixer
- Agilent Bioanalyzer or similar device - optional

Consumables

- QIAcuity Nanoplate 26k 24-well (QIAGEN, Cat.#: 250001)
- PCR microplate, 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
- 2 ml reaction tubes
- 0.5 ml low protein binding tube (e.g. Eppendorf, Cat.#: 0030108094)
- PCR tubes - optional
- 15 ml falcon tubes
- 50 ml falcon tubes
- Syringes (5 ml and 20 ml)
- 10 µl, 200 µl, 1,000 µl standard pipette tips
- Aluminum foil
- Reagent reservoirs

Chemicals and Kits

- PICOglue BL, P8, N6, or O7 Label (Cat.#: PICO-000120 - 123)
- PICO AMC Kit (Cat.#: PICO-000010)
- PICO BL, P8, N6, or O7 Probe (Cat.#: PICO-000070 - 73)
- 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)
- Agilent Protein 230 kit (Agilent, Cat.#: 5067-1517) - optional
- Suitable antibody *(see 06.1 Antibody Requirements)*

08 Protocol of the PICOglue Antibody Labeling Kit

Note: The protocol is valid for one antibody labeling reaction. Do not adjust reagent volumes based on variations in sample volume.

08.1 Rebuffering of the Antibody

1. Prepare 2 ml of 1x Ultrafiltration Buffer by diluting the 10x Ultrafiltration Buffer (stored at 4°C) with ultrapure water.
   The 1x Ultrafiltration Buffer can be stored at 4°C for one week and will also be used at *Section 08.4 step 22*.

2. Vortex the antibody for 5 s and spin down (~1,000 rcf, 5 s). Place a 100K Ultrafiltration Column into a Collection Tube (**Figure 2a**). Load 50 - 100 µg of antibody into the Ultrafiltration Column (see *06.2 Concentration of Antibodies*).
   Maximum 400 µl of antibody solution can be loaded into the Ultrafiltration Column. If the volume exceeds 400 µl, add it to the column in several steps and centrifuge in between for 3 min (14,000 rcf, 4°C). Discard flow-through after each centrifugation.

3. Wash the antibody by adding up to 400 µl of 1x Ultrafiltration Buffer to the Ultrafiltration Column and centrifuge at 14,000 rcf for 3 min at 4°C. Discard flow-through.
   The same Collection Tube can be used for all washing steps.

4. Repeat the washing step once more by adding up to 400 µl of 1x Ultrafiltration Buffer and centrifuge at 14,000 rcf for 10 min at 4°C.

5. Place a new Collection Tube over the Ultrafiltration Column and invert it. (**Figure 2d**). Recover the antibody using a quick spin (~1,000 rcf, 5 s) (**Figure 2e**). Discard the Ultrafiltration Column.

6. Transfer the recovered antibody to a new 1.5 ml reaction tube.
   Record the volume of the recovered antibody. The acceptable range of recovered antibody volume is 15 - 50 µl. In this manual, deglycosylation and azide attachment is carried out in a 1.5 ml reaction tube as an example. However, the reactions can also be carried out in a PCR cycler using PCR tubes.

7. If intended, save 1 µl of antibody solution as ‘unconjugated control’ for checking conjugation efficiency (see *Section 10.1*). Store the unconjugated control at 4°C until further use.

08.2 Deglycosylation of the Antibody

8. Pre-warm the heating block to 37°C.

9. Add 3 µl of 10x PICOzyme Buffer (stored at -20°C) and 3 µl of PICOzyme (stored at 4°C) to the antibody solution, recovered in *Section 08.1 step 5*. Fill up to a total reaction volume of 30 µl with 1x Ultrafiltration Buffer, mix by vortexing for 5 s, and spin down (~1,000 rcf, 5 s).

If the total reaction volume exceeds 30 µl, due to the high volume of recovered antibody, do not add additional 1x Ultrafiltration Buffer.

10. Place the tube into the pre-warmed heating device. Incubate for 1 h at 37°C.

11. After incubation, vortex for 5 s, and spin down (~1,000 rcf, 5 s).

12. Determine the volume of the antibody solution and store the antibody at 4°C until proceeding to *Section 08.3 step 16*.

13. If intended, save 1 µl of antibody as 'deglycosylated control' for analysis of the deglycosylation process (see *Section 10.1*). Store the deglycosylated control at 4°C until further use.

08.3 Azide Attachment

14. Pre-warm the heating block to 30°C.

15. Thaw one vial of PICOtransferase Substrate (stored at -20°C). Reconstitute it with 10 µl of ultrapure water and incubate for at least 20 min at room temperature. Vortex for 5 s and spin down (~1,000 rcf, 5 s).

16. In a new 1.5 ml reaction tube, mix the antibody solution from *Section 08.2 step 12* and the reconstituted 10 µl of PICOtransferase Substrate. Add the following reagents to this tube:

    5 µl 10x PICOtransferase Buffer A (stored at 4°C)
    5 µl 10x PICOtransferase Buffer B (stored at 4°C)

17. Fill up to a total reaction volume of 48.7 µl with ultrapure water. Mix by vortexing for 5 s and spin down (~1,000 rcf, 5 s).
    If the total reaction volume exceeds 50 µl, due to the high volume of recovered antibody, do not add additional ultrapure water.

18. Add 1.3 µl of PICOtransferase (stored at 4°C), mix by vortexing for 5 s, and spin down (~1,000 rcf, 5 s).

19. Place the tube into the pre-warmed heating device. Incubate overnight at 30°C protected from light.
    The incubation period can vary between 12 and 24 hours. If the tube is not suitable for the heating device, the solution can be transferred to a 0.5 ml reaction tube or a 30°C incubator can be also used instead.

08.4 Cleaning up the Antibody

20. After overnight incubation, vortex for 5 s, and spin down (~1,000 rcf, 5 s).

21. Place a 100K Ultrafiltration Column into a Collection Tube (**Figure 2a**).

22. Transfer the antibody solution to the 100K Ultrafiltration Column and wash by adding up to 400 µl of 1x Ultrafiltration Buffer. Centrifuge at 14,000 rcf for 3 min at 4°C. Discard flow-through.
    The 1x Ultrafiltration Buffer prepared at *Section 08.1 step 1* can be used here. The same Collection Tube can be used for all washing steps.

23. Repeat the washing step once more by adding up to 400 µl of 1x Ultrafiltration Buffer. Centrifuge at 14,000 rcf for 10 min at 4°C. Discard flow-through.

24. Place a new Collection Tube over the Ultrafiltration Column and invert it (**Figure 2d**). Recover the antibody using a quick spin (~1,000 rcf, 5 s) (**Figure 2e**). Discard the Ultrafiltration Column.
    The acceptable range of recovered antibody volume is 15 - 50 µl.

08.5 Label Attachment

25. Transfer the recovered antibody to a new 1.5 ml reaction tube. Add 4 µl of the PICOglue Label (P8, BL, N6, or O7), mix by vortexing for 5 s, and spin down (~1,000 rcf, 5 s). Incubate overnight at 4°C protected from light.
    The incubation can be extended up to 48 hours.

26. After overnight incubation, if intended, save 1 µl of the antibody solution as 'labeled antibody' for analysis of the labeling efficiency with Agilent's Bioanalyzer system (see *Section 10.2*).
    Optional: to perform the analysis of antibody deglycosylation and labeling efficiency with Agilent's Bioanalyzer skip ahead to *Section 10*. We recommend performing this before proceeding with the removal of the free labels.

08.6 Removal of Free Labels

Note: If multiple antibodies are labeled in parallel, we recommend performing the removal of free labels separately for each antibody. Thoroughly clean the workbench and equipment in between due to the high risk of cross-label contamination.

27. After overnight incubation, vortex for 5 s and spin down (~1,000 rcf, 5 s).

28. Prepare 6 ml of 1x Wash Buffer I in a 50 ml falcon tube by diluting 2x Wash Buffer I with ultrapure water. Set up another 50 ml falcon tube as a waste vessel.
    The 1x Wash Buffer I can be stored at 4°C for one week.

29. Place a 100K Ultrafiltration Column into the provided Collection Tube (**Figure 2a**).

30. Transfer the antibody solution to the 100K Ultrafiltration Column and wash by adding up to 400 µl of 1x Wash Buffer I. Centrifuge at 14,000 rcf for 3 min at 4°C. Discard flow-through.
    The same Collection Tube can be used for all washing steps.

31. Repeat the washing step once more by adding up to 400 µl of 1x Wash Buffer I and centrifuge at 14,000 rcf for 10 min at 4°C.

32. Place a new Collection Tube over the Ultrafiltration Column and invert it (**Figure 2d**). Recover the antibody using a quick spin (~1,000 rcf, 5 s) (**Figure 2e**). Discard the Ultrafiltration Column. Fill up to a total volume of 40 µl using 1x Wash Buffer I.
    The acceptable range of recovered antibody volume is 15 - 50 µl. If the total reaction volume exceeds 40 µl, due to the high volume of recovered antibody, do not add additional 1x Wash Buffer I .

33. Break off the nozzle of the Loading Column and place it into a 1.5 ml reaction tube **(Figure 3a)**.

34. Homogenize the PICOglue Antibody Binding Resin (stored at 4°C) by vortexing carefully for 5 s. Add 100 µl of resin to the Loading Column ensuring a thin coverage over the filter.
    Cutting the tip of the pipette tip and careful mixing by pipetting up and down five times ensures optimal resin transfer to the Loading Column. Avoid any unnecessary incubation time to avoid settling of the resin. At this stage, the thin resin layer may not be clearly visible.

35. Close the lid leaving the cap open and attach a syringe filled with 2 ml 1x Wash Buffer I (+ air) (**Figure 3b**). Wash carefully with gentle but constant pressure into the waste vessel. Detach the syringe and transfer the Loading Column into a new 1.5 ml reaction tube.
    Always load additional 1 ml volume of air in the syringe to remove excess liquid from the Loading Column. After the first washing step the thin resin layer becomes barely visible.

36. Repeat the washing step once more with 2 ml 1x Wash Buffer I (+ air). Transfer the Loading Column into a new 1.5 ml reaction tube and spin down (~1,000 rcf, 5 s).
    After spinning, the resin might be visible as a tilted surface (**Figure 3b**). Avoid any unnecessary incubation time to prevent the resin from drying out.

37. Pipette the antibody solution, recovered in *step 32,* into the Loading Column by avoiding touching the tube (**Figure 3c**). The resin will soak up the volume. Incubate for 1 h at room temperature leaving the Loading Column in the 1.5 ml reaction tube.
    Add the antibody solution immediately to prevent the drying out of the resin.

38. Prepare 25 ml of 1x Wash Buffer II by diluting the 2x Wash Buffer II with ultrapure water. Set up a new 50 ml falcon tube as a waste vessel.
    The 1x Wash Buffer II can be stored at 4°C for one week.

39. Attach a syringe loaded with 7 ml (+ air) of 1x Wash Buffer II to the Loading Column. Wash carefully with gentle but constant pressure into the waste vessel (**Figure 3b**). Detach the syringe and transfer the Loading Column into a new 1.5 ml reaction tube.
    Always load additional 1 ml volume of air in the syringe to remove excess liquid from the Loading Column. Always transfer the Loading Column to a new reaction tube after every wash to prevent liquid contamination from the previous washing step.

40. Repeat the washing step twice more with 7 ml (+ air) of 1x Wash Buffer II. Transfer Loading Column into a new 1.5 ml reaction tube after each washing step.
    Be careful when washing the Loading Column. The high concentration of PICO Labels can contaminate the laboratory environment. If working simultaneously with antibodies conjugated with different PICO Labels, we recommend changing gloves and cleaning the working area in between the washing steps to avoid cross-label contamination.

41. Transfer the Loading Column into a new 1.5 ml reaction tube and spin down to remove the last traces of the buffer (~1,000 rcf, 5 s).

42. Transfer the Loading Column into a new 2 ml reaction tube and add 300 µl of PICOglue Elution Buffer (◯ stored at 4°C) to the resin. Carefully mix by pipetting and incubate for 30 min at room temperature leaving the Loading Column in the 2 ml reaction tube.
    Use a 2 ml reaction tube, otherwise the volume of the tube will not be sufficient for the recovery volume at the next step. Add the Elution Buffer immediately to prevent drying out of the resin.

43. Recover the labeled antibody using a quick spin (~1,000 rcf, 10 s). Discard the Loading Column.

08.7 Rebuffering of the Antibody

44. Transfer the labeled antibody into a new 100K Ultrafiltration Column (**Figure 2a**). Fill the column up to 400 µl with PBS and centrifuge at 14,000 rcf, 3 min at 4°C. Discard flow-through.
    The same Collection Tube can be used for all washing steps.

45. Add 400 µl of PBS to the Ultrafiltration Column and repeat the centrifugation at 14,000 rcf for 10 min at 4°C. Discard flow-through.

46. Place a new Collection Tube over the Ultrafiltration Column and invert it (**Figure 2d**). Recover the antibody using a quick spin (~1,000 rcf, 5 s) (**Figure 2e**).

47. Transfer the antibody to a low protein binding tube.
    The range of recovered antibody volume is normally between 15 to 50 µl.

48. For optimal storage conditions add an appropriate amount of 10x PICOglue Antibody Storage Buffer (● stored at -20°C) and mix by vortexing for 5 s, and spin down (~1,000 rcf, 5 s).
    Adding the appropriate amount of 10x PICOglue Antibody Storage Buffer results in a 1:10 dilution. For example, if you recover 27 µl antibody solution, add 3 µl of 10x PICOglue Antibody Storage Buffer. The labeled antibodies can be stored at 4°C and used for at least 12 months. Use low binding tubes for storage and handle sterile to increase stability.

09 Quality Control of Labeled Antibody

Note: Quality Control of a labeled antibody has to be performed only once for each newly labeled antibody. The reagents and chemicals from the PICO AMC Kit and PICO BL, P8, N6, or O7 Probes are used here. The protocol is valid to control the quality and determine the concentration of one labeled antibody. However, more than one antibody can be analyzed in parallel. We recommend measuring at least three replicates for each antibody dilution sample in the dPCR.

09.1 Buffer Preparation

1. Prepare the chemicals and buffers as listed below.
   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 dissolved Additive C
80 µl PIC
200 µl Additive L
120 µl PBS
Stable for 3 days at 4°C

Control Buffer (CB)
250 µl LB-Stock (2x stock)
100 µl BSA
150 µl PBS
Prepare the Control Buffer fresh

PICOglue Antibody Storage Buffer
20 µl 10x PICOglue Antibody Storage Buffer
180 µl PBS
Stable for 6 months at 4°C
For long-term storage we recommend adding an additional 8.3 µl of 25x PIC solution.

09.2 Preparation for dPCR

2. Prepare the Master Mix for 24 samples. Make sure that the Master Mix contains the probe needed (corresponding to the PICO Label used for labeling). If another antibody with a different label is analyzed, add the additional probe to the same Master Mix by replacing 45 µl of ultrapure

water with the same volume of the corresponding PICO Probe (see amounts below). Vortex for 10 s and spin down (~1,000 rcf, 5 s).

Store the Master Mix at 4°C until used.

Master Mix	
Reagents	**Volume**
Ultrapure water	742 µl
QIAcuity Probe Master Mix	284 µl
PICO Probe (P8, BL, N6, or O7)	45 µl
Coupling dPCR Mix	36 µl

3. Dilute the labeled antibody 1:40 in PICOglue Antibody Storage Buffer in a low protein binding tube (**Figure 4a**). Mix by vortexing for 10 s and spin down (~1,000 rcf, 5 s). Sonicate for 1 min at full power in an ultrasonic bath and spin down (~1,000 rcf, 5 s).

   This is defined as dilution step 1 (DS1) and can be stored as a working stock for PICO experiments.

4. Add 39 µl of CB to a reaction tube and transfer 1 µl of diluted antibody (DS1) to it (**Figure 4a**). Sonicate for 5 min at full power in an ultrasonic bath and spin down (~1,000 rcf, 5 s).

   This is defined as dilution step 2 (DS2).

5. Add 39 µl of PBS in four additional reaction tubes. Starting from DS2, prepare a serial dilution by transferring 1 µl of labeled antibody from one reaction tube to the next (DS2 to DS6). Mix by vortexing for 5 s and shortly spin down (~1,000 rcf, 2 s) between each dilution step (**Figure 4a**).

   These are defined as dilution steps 3-6 (DS3 - 6).

6. Add 41 µl of Master Mix into columns 1 - 3 of a 96-well plate.

7. Transfer 1 µl from DS4-6 into the corresponding B1 - H3 wells. Leave well A1 filled with Master Mix only for non template control (NTC). Mix by pipetting up and down five times (**Figure 4b**). Avoid the formation of air bubbles.

09.3 dPCR and Evaluation

8. Transfer 40 µl from each well containing the Master Mix and the diluted antibody (columns 1 - 3) to 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. Set up the dPCR program in the QIAcuity Software Suite using the following dPCR settings:

   An example screen recording for setting up a Nanoplate in the QIAcuity Software Suite can be watched here.

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

9. After the dPCR run, open 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'.

10. 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 after adjusting the threshold for a channel, the values have to be recalculated.

11. After adjusting the thresholds, select 'List', click 'Export to CSV…' and select 'Current results'.

    Please check the raw data and images of the plate in the QIAcuity Software Suite to ensure that the data is valid and that the dilution series was performed correctly.

12. Calculate the lambda (λ) (average number of detected targets per partition) of each sample.

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

    For further evaluation, consider only the results of the dilution step in which the samples have a λ between 0.01 and 5. Usually, only one dilution will be in the valid range. If two dilutions are valid, consider the dilution with the smaller lambda.

13. Calculate the antibody concentration using the Antibody Concentration Calculator. To calculate it manually follow the steps in *section 11.1*.

10 Analyzing Antibody Deglycosylation and Labeling Efficiency - optional

Note: The peak sizes described in this manual can vary depending on the antibody and the individual run on the Agilent Bioanalyzer. To analyze the antibody deglycosylation and labeling efficiency using the Agilent 2100 Expert software additional manual changes might be necessary.

If the software does not detect a peak, use the 'Add-Peak' function. Right click on the electropherogram and choose 'Manual Integration'. Then right click on the electropherogram at the position of the peak that needs to be added and press 'Add-Peak' to determine the top and bottom of the peak. If the system peaks ('Upper Marker' or 'Lower Marker') are not set correctly, right click on the corresponding peak in the peak table and manually set the upper or lower maker.

1. Agilent Protein 230 Kit contains the chip and reagents designed for analysis of proteins. Run the antibody samples according to the manufacturer instructions.

10.1 Antibody Deglycosylation Efficiency

2. To investigate the deglycosylation of the antibody (*Section 08.2*), 1 µl of the deglycosylated antibody (*Section 08.2 step 13*) is used. Additionally, the 'unconjugated control' (*Section 08.1 step 7*) is also required.

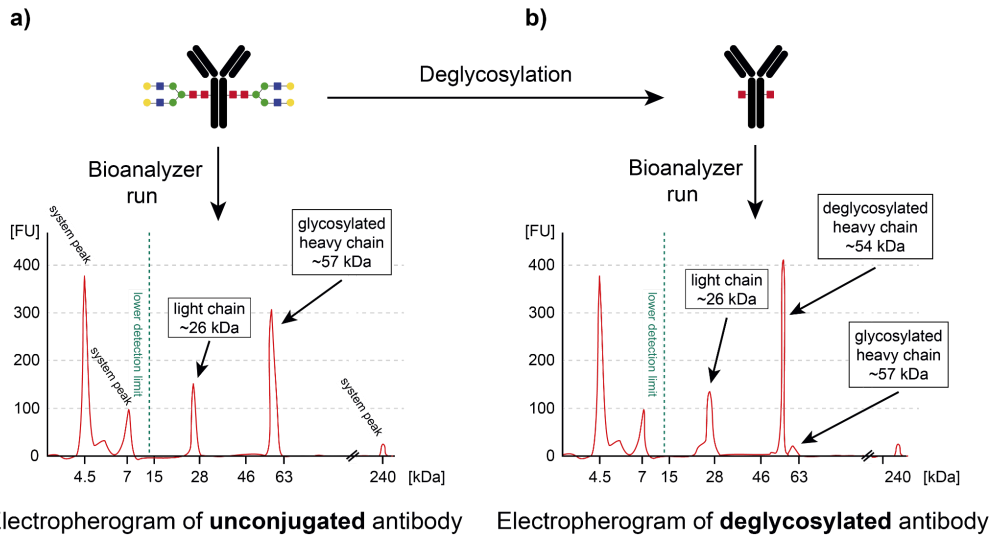


Figure 5. Electropherogram of unconjugated and deglycosylated antibodies.

3. **Figure 5** shows the relevant peaks to evaluate the deglycosylation process. To measure the deglycosylation efficiency (DE), record the relative concentration [ng/µl] of the following peaks (see **Figure 5** as a reference):

   26 ± 3 kDa – light chain (lc)
   50 ± 4 kDa – deglycosylated heavy chain ($hc_{deglycosylated}$)
   54 ± 4 kDa – native heavy chain (hc_{native})

4. Copy the concentrations of the peaks into the corresponding cells of the 'Deglycosylation and Labeling Efficiency Calculator' to calculate the deglycosylation efficiency (DE) automatically.

   The deglycosylation efficiency is calculated as below. The deglycosylated and unmodified peaks of the heavy chain of the antibodies are used (rel. conc. [ng/µl]) (see example in **Figure 5b**) according to:

$$DE = \left(\frac{[hc_{deglycosylated}]}{[hc_{deglycosylated} + hc_{native}]} \right) \times 100$$

   Please note that a low DE will lead to low labeling efficiency and the antibody might not be suitable for the PICOglue labeling technology. In this case, contact us for a different labeling technology(see *12 Ordering Information*). In case of unclear results, see troubleshooting guide.

10.2 Antibody Labeling Efficiency

5. To analyze the labeling efficiency of the antibody, 1 µl of the labeled antibody (*Section 08.5 step 26*) is used.

6. **Figure 6** shows the relevant peaks to evaluate the labeling efficiency. To measure the labeling efficiency (LE), record the rel. conc. [ng/µl] of the following peaks (see **Figure 6** as a reference):

   26 ± 3 kDa – light chain (lc)
   50 ± 4 kDa – unlabeled heavy chain ($hc_{unlabeled}$)
   75 ± 5 kDa – heavy chain + label ($hc_{labeled}$)

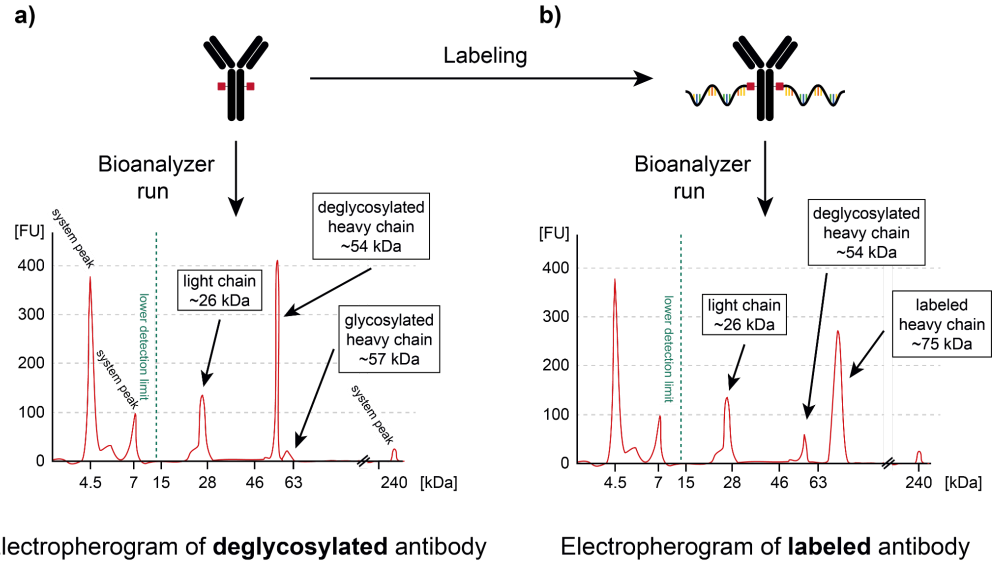


Figure 6. Electropherogram of deglycosylated and labeled antibodies.

7. **Figure 6** shows the relevant peaks for determination of the labeling efficiency. Copy the concentrations of the peaks into the corresponding cells of the 'Deglycosylation and Labeling Efficiency Calculator' to calculate the labeling efficiency automatically.

   The labeling efficiency is calculated as below. The labeled and unlabeled peaks of the heavy chain of the antibodies are used (rel. conc. [ng/µl]) (see example in **Figure 6b**) according to:

$$LE = \left(1 - \left(\frac{[hc_{unlabeled}]}{[hc_{unlabeled} + hc_{labeled}]}\right)^2\right) \times 100$$

Please keep in mind that the unlabeled fraction of the heavy chain can consist of two peaks derived from a deglycosylated fraction and a native glycosylated fraction. In this case, the sum of both peaks are used as unlabeled heavy chain for the calculation.

8. The expected labeling efficiency is above 70%. In case of lower labeling efficiency or unclear labeling results, see troubleshooting guide.

11 Appendix - Calculations

11.1 Calculation of the Antibody Concentration

1. Calculate the dilution factor introduced by the serial dilution of the antibody stock and the Master Mix volume.

$$Dilution\ factor\ =\ 42\ \times\ 40^{\#DS}$$

$42:$ *Last dilution step introduced by the volume of the Master Mix*

$\#DS:$ *Number of dilution step of the dilution sample used for the calculation* (*e. g.* 4 *for DS*4)

2. Calculate the antibody stock concentration [cp/µl] using the antibody concentrations of the dilution step within the valid range of λ (see *step 10*). The average of these antibody concentrations is multiplied by the dilution factor.

$$Antibody\ stock\ conc.=\ average(antibody\ conc.)\ \times\ dilution\ factor$$

Example: The lambda of the four times serial diluted sample (DS4) is in the range between 0.01 and 5. Calculate the average of the measured concentrations of all replicates (e.g. 200 cp/µl) of this antibody dilution. The average obtained antibody concentration is multiplied with 42×40^4 (107,520,000) resulting in a stock concentration of 2.15×10^{10} cp/µl.

12 Troubleshooting Guide

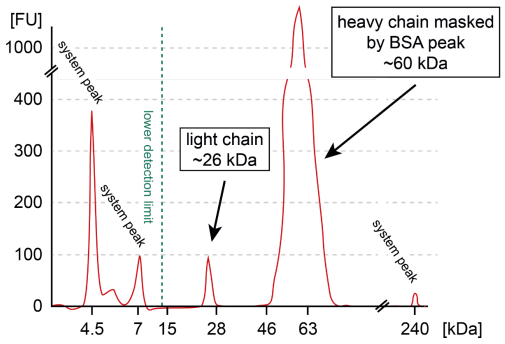
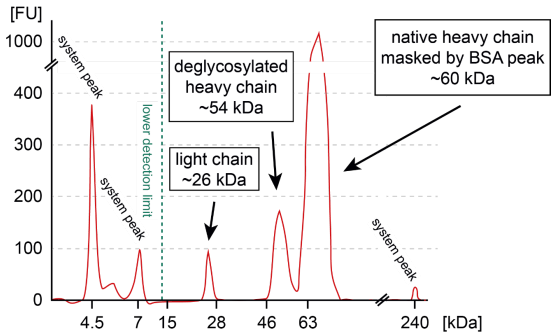
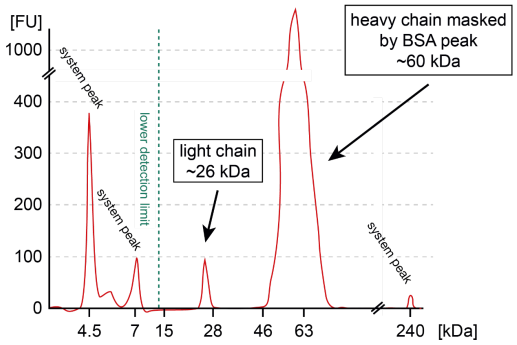
Consult the troubleshooting guide below to solve any problems that may arise. For more information, see the Frequently Asked Questions (FAQ). It is available at: www.actome.de/resources/downloads

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

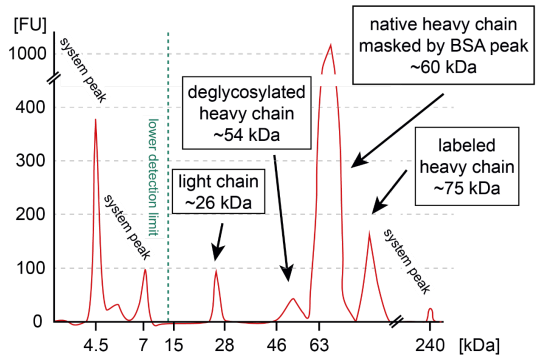
Troubleshooting

Issue	Comments and Suggestions
The recovered volume after rebuffering is larger than expected	
The required rcf is not met	Increase the time of centrifugation by up to 30 min.
Low labeling efficiency (<70%)	
Repeatedly low labeling efficiency	Make sure to use high quality antibodies.
Antibody is not susceptible to deglycosylation	Analyzing a sample after deglycosylation (*Section 08.2 step 12,* see *section 10*) provides information about whether the antibody was successfully deglycosylated. If not, the antibody might not be suitable for labeling using PICOglue technology. In this case please contact us for alternative solutions.
All partitions detected in dPCR of quality control of labeled antibodies are positive	
Dilution of antibodies was too low	Increase the dilution factor of the antibodies by 40
No signal detected in dPCR of quality control of labeled antibodies	
Dilution of antibodies was too high	Reduce the dilution factor of the antibodies by 40.
Unclear peaks in the electropherogram during analysis of the deglycosylation efficiency	
Heavy chain of the antibody is masked by BSA	The electropherogram can show peaks from the buffer of the antibody such as BSA masking the heavy chain as depicted left. In this case, the deglycosylation efficiency cannot be calculated, but this does not affect the labeling reaction itself.

Troubleshooting

Issue	Comments and Suggestions
Unclear peaks in the electropherogram during analysis of the deglycosylation efficiency	
Heavy chain of the antibody is masked by BSA Electropherogram of **unconjugated** antibody  Electropherogram of **deglycosylated** antibody  	The electropherogram can show peaks from the buffer of the antibody such as BSA masking the heavy chain as depicted left. In this case, the deglycosylation efficiency cannot be calculated, but this does not affect the labeling reaction itself.
Unclear peaks in the electropherogram during analysis of the labeling efficiency	
Heavy chain of the antibody is masked by BSA Electropherogram of **unconjugated** antibody  	The electropherogram of an antibody can contain peaks from disruptive factors such as BSA masking the heavy chain as depicted left (unconjugated antibody) but this does not affect the labeling reaction itself. In the electropherogram of the labeled antibody, the deglycosylated heavy chain can either be detected as depicted in the upper image or masked by the BSA peak (see lower image). To evaluate the labeling efficiency in the latter case, it can be estimated that an antibody contains twice the concentration (rel. conc. [ng/µl]) of heavy chains than of light chains. With this consideration, the labeling efficiency can be roughly estimated using the concentration of the light chain (lc):

Electropherogram of **labeled** antibody



$$LE = \left(1 - \left(\frac{\left[2 \times lc - hc_{labeled}\right]}{[2 \times lc]}\right)^2\right) \times 100$$

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 PICO Product		
PICOglue Antibody Labeling Kit	Labeling of antibodies with PICOglue technology (4 rxns)	PICO-000110
Related PICO Products		
PICOglue Labels	BL (HEX), P8 (FAM), N6 (TAMRA), O7 (ROX) label for antibody labeling (4 rxns)	PICO-000120 - 123
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