actome

## *What is PICO?*

The Protein Interaction Coupling technology!

# Frequently Asked Questions

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# General

- **What is PICO?**

  Protein Interaction Coupling (PICO) is an ultra sensitive immunoassay for the detection and absolute quantification of proteins, protein interactions, and post-translational modifications. PICO combines the advantages of immunoassays with digital PCR technology, by translating protein status into DNA barcodes.

- **What can PICO measure?**

  With PICO you can measure single proteins, protein interactions, and post-translational modifications from any soluble biological sample. For a consistent nomenclature, we call them 'targets'.

- **What are the requirements for PICO?**

  For the PICO assay, a pair of antibodies is required against each target. The two antibodies have to bind to non-overlapping and concurrently accessible epitopes.

- **How does PICO work in a nutshell?**

  The PICO workflow has four major steps:

  1. **Antibody Labeling**
     - Two different monoclonal antibodies of your choice are labeled with unique DNA oligonucleotides (PICOglue Labels).
  2. **PICO Assay**
     - The biological sample is lysed, mixed, and incubated overnight with the labeled antibodies. '*Couplexes*', a target bound by the two different labeled antibodies, are formed.
     - After incubation, the sample is highly diluted, mixed with the dPCR Master Mix and the matching PICO Probes, and then transferred to a dPCR assay plate.
  3. **dPCR Amplification**
     - In the dPCR assay plate, the sample is partitioned into thousands of compartments.
     - During amplification, specific fluorescent signals are generated that are detected by the dPCR instrument.
     - Compartments containing the couplexes are counted.
  4. **Data Analysis**
     - The raw dPCR data is uploaded and analyzed by AMULATOR, Actome's online data analysis software.
     - The number of couplexes are converted into precise molar concentrations.

- **What are the advantages of PICO compared to other protein detection assays?**

  PICO is designed to outperform existing protein detection and quantification solutions (e.g. western blot, co-immunoprecipitation (coIP), ELISA, or proximity ligation assay (PLA)) and to introduce powerful experimental possibilities:

  - **Absolute quantitative:** PICO enables the absolute quantification of protein targets without the need of an external standard. The PICO data provides precise molar concentration of the target protein, protein interaction, or post-translational modification.
  - **Ultra-high sensitivity:** The limit of detection (LOD) of PICO is in the femtomolar range, meaning with high quality antibodies you can detect 100 target molecules per cell. Due to the ultra-high sensitivity, the target protein can be detected from as low as 1,000 cells.
  - **Zero background:** PICO doesn't have any background reactions. During the dPCR step, the sample is compartmentalized to exclude the possibility of target-independent background reactions. Therefore, there is zero signal in case no target is present in the dPCR reaction.

- **Low sample input:** The PICO assay only requires 2 µl of sample as an input, which is significantly lower than the sample requirements for western blot, coIP, or ELISA. Thus, together with ultra-high sensitivity, you can save on precious biological material.
- **Multiplex assays:** The dPCR instruments can often detect at least five fluorescent colors, meaning five different antibodies with different PICOglue Labels. Thus, PICO offers extensive flexibility in designing parallel assays.

## What is the sensitivity of PICO?

The sensitivity of PICO is highly dependent on the quality (dissociation constant, $K_D$) of the antibodies. Using high quality antibodies ($K_D$ in the $10^{-11}$ - $10^{-13}$ range) allows measurements in the ~100 - 500 fM protein concentration range. Average antibodies ($K_D$ in the $10^{-9}$ - $10^{-11}$ range) enables detection in the 1 - 5 pM concentration range.

## How much more sensitive is PICO compared to western blot?

Comparing the detection of the breast cancer oncoprotein HER2 with western blot and PICO, revealed that PICO is approximately 200-fold more sensitive than a western blot.

## What are the requirements/recommendations concerning antibodies?

The PICO assay works best with monoclonal antibodies since they are often better characterized and defined by the antibody producer and the batch-to-batch variability is minimal. As the antibodies need to bind concurrently, choosing monoclonal antibodies raised against different epitopes on the protein is critical.

## What is the underlying molecular biological principle of PICO?

In a PICO assay, protein levels are translated into DNA barcodes by using DNA-labeled antibodies. First, the biological sample, containing the target protein, protein interaction or post-translational modification, is incubated with two different DNA-labeled antibodies. During the incubation, the antibodies bind to their target(s) and form a complex, termed 'couplex', which is the detection unit of the PICO assay. After overnight incubation the samples are highly diluted, partitioned, and analyzed by digital PCR (dPCR). During the dPCR reaction, specific probes bind to the DNA-labels on the antibodies. As the amplification occurs, the probes are hydrolysed and a distinct fluorescent signal is generated and recorded by the dPCR instrument. Partitions giving rise to both fluorescent colors, containing the couplexes, are counted. Based on the statistical distribution of the fluorescent signal in zero, single, or double positive partitions, Actome's online AMULATOR software converts the number of couplexes to molar concentrations.

## What is a couplex?

A couplex is a target bound by two labeled antibodies and is the molecular detection unit of the PICO assay.

## What is the underlying mathematical assumption of PICO?

The key step in the analysis of PICO data is differentiating genuine, couplex-containing partitions from the double-positive partitions that arose from random co-occurrence. A statistical algorithm was developed to analyze the number of partitions in a dPCR reaction that showed zero, single, or double positive fluorescent signals. It is recommended that each experiment include an antibody binding control (ABC) sample. The ABC sample contains the same concentration of labeled antibodies as in the sample-containing wells, but lacks a biological sample (antigen). The labeled antibodies in the ABC sample are distributed randomly, following a Poisson distribution, among all partitions. Consequently, the cluster of double-positive partitions in the ABC is a direct consequence of the overlapping antibody populations, resulting in zero couplexes in the ABC sample. The presence of the biological target (antigen) in the samples results in an enrichment of double positive partitions, a deviation from the Poisson distribution. Actome's algorithm is capable of distinguishing between random double positive partitions and those containing couplexes, and enables the conversion of couplex amounts to molar concentrations.

- **Why are two antibodies necessary to detect a target?**

  With a single antibody it is impossible to distinguish the target-bound and unbound antibodies in the dPCR reaction. However, using two DNA-labeled antibodies will generate a double-positive signal in a dPCR partition, which can be distinguished from random double-positive partitions by the AMULATOR algorithm.

- **What is digital PCR and what are the advantages?**

  In comparison to a standard PCR or quantitative PCR (qPCR), during digital PCR (dPCR) the sample is partitioned into thousands of minuscule partitions before amplification. In each partition the template is independently amplified and fluorescent signal is generated, allowing the detection of the partitions containing a template (end-point PCR). A high dilution of the sample prior to the partitioning, ensures that not all partitions contain templates and thus negative partitions, which are necessary for mathematical evaluation, are also present. By counting all the individual positive partitions, dPCR enables the absolute quantification of target molecules without the need for an external reference. In addition, the digital nature and the resistance to PCR inhibitors elevates dPCR over the classical PCR or qPCR.

- **What are the underlying mathematical assumptions of dPCR?**

  Positive partitions in dPCR (partitions containing amplified DNA template) are detected by the generated fluorescent signals. The distribution of the templates, during the partitioning step, follows Poisson statistics. Based on this Poisson distribution, the precise concentration of the template molecules in the whole reaction can be calculated. For more details, see Basu et. al 2017.

- **How much lab work is required for the PICO assay?**

  The PICO assay itself is a two-day process. On the first day, the sample is lysed and combined with the labeled antibody mix for overnight incubation. The next day the sample is highly diluted, mixed with the dPCR Master Mix and the PICO Probes, and analyzed by dPCR. The total hands-on time is approximately ~1h 30 min.

- **What is the 'binding reaction'?**

  The binding reaction is the mixture of the labeled antibodies and the biological sample. It is incubated at 4°C overnight to enable the formation of couplexes (detection unit of the PICO assay).

- **Which devices are required to perform a PICO assay?**

  The PICO assay only requires a digital PCR instrument beside standard laboratory equipment such as, multi-channel pipettes, centrifuges, vortex, and an ultrasonic bath. Optionally, Agilent's Bioanalyzer is required to determine the antibody conjugation efficiency during the PICOglue Antibody Labeling workflow.

- **Is there an alternative solution if no ultrasonic bath is available?**

  Ultrasonic bath is used for homogenizing the samples, especially when the sample volumes are very small. In case an ultrasonic bath is not available, the sonication can be replaced by carefully pipetting up and down.

- **What are the safety precautions for using PICO kits?**

  Follow general safety precautions and wear protective gear (lab coat and gloves). Do not eat, drink, or inhale any substances or apply them on skin/eye. If skin or eyes come in contact with any substances, rinse thoroughly. In case of exposure to any chemicals seek immediate medical attention.

- **Where can I purchase the PICO kits?**

  The PICO kits are available for purchase in Actome's webshop or write to us at sales@actome.de to request a quote.

# Technical

- **What is the laboratory workflow of a PICO assay?**
  1. Lysis of the biological sample. The PICO assay can be performed using different biological sample types such as, lysed cells, supernatant, blood, or plasma.
  2. Preparation of the antibody mix (ABX), containing the labeled antibodies in a specific concentration, to ensure optimal sensitivity and precision of the assay.
  3. Mixing of the biological sample with the ABX and overnight incubation (termed 'binding reaction'). During the binding reaction the couplexes are formed.
  4. High dilution and mixing of the binding reaction with the dPCR Master Mix and the PICO Probes. The diluted samples are loaded onto the dPCR plate and the dPCR amplification is performed.
  5. The raw dPCR data is exported and evaluated with Actome's online AMULATOR software
- **What is an ABC?**

  ABC (antibody binding control) is a control sample containing only the labeled antibody mixture (ABX) without the biological sample (antigens). Ideally, after the AMULATOR calculates the number of couplexes, the ABC yields zero couplexes because the expected Poisson statistical overlap is subtracted. However, due to drop-out events and statistical offsets, the ABC can deviate from zero and thus the sample couplex count has to be normalized to the ABC couplex count.
- **What is a NTC?**

  NTC (non template control) is a sample without any template DNA or antibodies, and serves as a quality control to assure correct and careful laboratory practice. Digital PCR is a highly sensitive technology and during the PICO workflow, reagents with high concentrations of DNA are handled. Therefore a NTC is necessary to confirm that no accidental contamination has occurred.
- **Why are no washing steps required in the PICO assay?**

  Actome's PICOglue Labeling Technology (carried out with the PICOglue Antibody Labeling Kit) workflow efficiently removes all DNA oligos (PICOglue Labels) that were not conjugated to the antibodies. Therefore, every detected signal during dPCR amplification is expected to originate from a labeled antibody. In addition, overnight incubation enables binding of the antibody to the target molecule at equilibrium concentration, allowing the maximum concentration of formed couplexes to be reached.
- **What happens to unbound antibodies after the binding reaction?**

  Unbound antibodies are pivotal for the couplex calculation in PICO as they determine the amount of random double positive partitions based on Poisson distribution. Therefore, unbound antibodies are not removed during the assay and are accounted for in the AMULATOR algorithm.
- **What additional consumables, chemicals, and devices are required during the PICO workflow?**

  **Consumables**
  - 1.5 ml reaction tubes
  - 2 ml reaction tubes
  - 0.5 ml low-protein binding tubes
  - Falcon tubes (15 ml, 50 ml)
  - PCR microplate, 96 well, V- and U-bottom
  - Sealing foil / adhesive film
  - dPCR assay plate

**Chemicals**

- PBS (without calcium or magnesium)
- cOmplete Protease™ Inhibitor Cocktail (Roche)
- RNAse free water
- dPCR Master Mix

**Devices**

- Regular (1 - 1000 µl) pipettes
- Multichannel pipettes, 8-channel (10 - 100 µl and 30 - 300 µl)
- Electronic multichannel pipette (1 - 12.5 µl) - *optional*
- Vortex mixer
- Table-top mini centrifuge
- Refrigerated Centrifuge
- Magnetic stand
- Ultrasonic bath
- dPCR instrument (QIAGEN's QIAcuity dPCR System, Stilla's naica® or Nio™)

- **What is lambda (λ) and how is it defined?**

  Lambda is defined as the average number of targets per partition. The average number of targets per partition (λ) depends on the sample concentration (C) and the partition volume ($V_p$), see Basu et al. 2017 for more detail.

  $\lambda = m/n = C * V_p$

# Biological Material

- **What type of samples can be used in a PICO assay?**

  Any biological sample such as, cell lysate, supernatant, FFPE samples, tissue, extracellular vesicles (EVs), blood serum, etc. can be analyzed with PICO. The samples must be liquefied, lysed, and homogenized, following the general laboratory instructions for a particular sample. In case of measuring cells (e.g. human cell lines) the lysis steps are included in the PICO Amplification Core Kit user manual.

- **What amount of cells are required for PICO?**

  We recommend using a concentration of 10,000 cells/µl and a total number of 20-40k cells to start with. However, due to different expression levels of proteins, the minimal amount of sample requirement also depends on the protein expression level.

- **What protein amount is necessary for PICO?**

  For high-expressing targets, we recommend starting with a total of 1-2 µg lysed cell material while for low-expressing targets, start with a total protein amount of 10 µg.

- **Can cryopreserved cells be used for PICO?**

  Yes, cryopreserved cells can be used for PICO assay.

- **How long can lysed cells be stored before running the PICO assay?**

  We recommend using lysed cells directly, however, the lysate can be stored at 4°C for up to 4 days. Additionally, cell lysate can be aliquoted and frozen at -20°C for at least a month.

- **Is it necessary to use a QIAshredder for the biological samples?**
  We recommend using the QIAshredder to homogenize the biological samples and our protocol is optimized based on that. If your sample does not need to be homogenized, this step can be omitted.
- **Can I use any other lysis buffer for my biological samples?**
  We recommend using the lysis buffer provided with our PICO Amplification Core Kit. In case you would like to use a lysis buffer of your choice, please contact our customer support team before proceeding with the PICO assay.

# Antibody Labeling

- **How much lab work is required for the antibody labeling?**
  The PICOglue Antibody Labeling Kit workflow is designed as a four-day procedure with an overall hands-on time of approximately 3 hours. The workflow can be paused at several time points, for example, to be resumed after a weekend break.
- **What is the shelf-life of the labeled antibodies?**
  The labeled antibodies, when stored in the 1x PICOglue Antibody Storage Buffer, are stable for at least 12 months at 4°C.
- **How is the concentration of the labeled antibodies determined?**
  The concentration of labeled antibodies is determined by dPCR using reagents from the PICO Amplification Core Kit together with matching PICO Probes. To determine the concentration of labeled antibodies follow the workflow 'Quality Control of the Labeled Antibody' as mentioned in the PICOglue Antibody Labeling Kit User manual.
- **What is an acceptable concentration for a labeled antibody?**
  In order to perform several PICO assays we recommend obtaining a labeled antibody concentration of at least $1 \times 10^9$ cp/µl. The higher the concentration of the labeled antibody, the more PICO reactions can be performed.
- **Is it possible to use the labeled antibodies for other applications?**
  Labeled antibodies can be used for other methods like western blot or coIP.
- **How long are the antibody dilutions stable?**
  If stored in 1x PICOglue Antibody Storage Buffer, dilutions of antibody stocks can be stored for up to six months at 4°C. 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 diluted) in 1x PICOglue Antibody Storage Buffer is recommended.
- **Can I reuse the antibody mix (ABX) for another PICO assay at a later time point?**
  If stored correctly at 4°C and protected from light, the ABX is stable for up to 3 weeks.

# AMULATOR

- **Where can I access the AMULATOR?**
  The AMULATOR can be accessed here: https://amulator.actome.de
- **Do I need any additional software for data analysis?**
  Microsoft Excel is required additionally for the calculation of the ABX and the sample dilutions.

- **What are the major steps during data analysis?**
  1. First-time users need to register an account first.
  2. After logging in, you can create projects where raw dPCR files, together with the sample definition file, are uploaded and analyzed. For each uploaded new dataset, a token is needed, which is provided with each PICO Protein Detection Trial Kit and PICO Amplification Core Kit.
  3. The data analysis takes approximately 5 minutes.
  4. The generated results (number of couplexes, lambda values) are presented in the form of a box plot graph, accompanied by fundamental statistical evaluations.
  5. In the projects overview section the data from previous experiments can be reviewed
- **Can I download my data and graphs from the AMULATOR?**

  You can download the graphs of the couplex and the lambda values from the option '*export graph images*'. The detailed data of the couplexes and lambda values can be downloaded from the option '*export data*'.

For further information visit www.actome.de and consult The PICO Handbook!

For any questions or inquiries don't hesitate to contact us at info@actome.de

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