Comparing HER2 Protein Amounts in BT474 and MCF7 Cells with PICO using Relative and Absolute Quantification

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Introduction

Breast cancer, a leading cause of cancer-related deaths in women, encompasses various subtypes, including HER2-positive breast cancer characterized by ERBB2 protein overexpression. Genomic amplification or overexpression of this oncogene drives aggressive tumor growth and metastasis. Anti-HER2 immunotherapy, utilizing antibodies like trastuzumab and pertuzumab, is a prominent treatment. Recently, the FDA approved Enhertu® as a therapeutic antibody-drug conjugate used in the treatment of HER2-positive breast cancer. It combines the anti-HER2 antibody trastuzumab with a cytotoxic payload to specifically target and kill HER2-overexpressing cancer cells. Enhertu® has demonstrated significant efficacy in clinical trials, however raised the necessity to detect HER2 cellular levels quantitatively also at low levels.

Highlights

- PICO enables absolute quantification of a target protein without the need of an external standard.
- PICO also enables relative quantification of target molecules between different samples or targets.
- BT474 cells contain ~120 fold more HER2 than MCF7 cells determined by relative quantification using PICO.

In research, immortalized breast cancer cell lines, such as HER2-positive BT474 and HER2-negative MCF7, are widely employed. While BT474 cells exhibit high HER2 expression detectable by common methods like western blot, detecting low HER2 levels remains challenging. Consequently, cell lines may be considered HER2-negative despite expressing HER2 at low levels. The Protein Interaction Coupling (PICO) technology is capable of detecting HER2 levels on low expressing cells as well (see this app note). In addition PICO enables the quantification of any target protein, protein interaction, and post-translational modification in both relative and absolute terms which is of great importance in both early-phase research development and in the diagnostic phase. In the following application note PICO was employed to compare the HER2 protein amounts in BT474 and MCF7 cells using relative quantification (RQ) and absolute quantification (AQ).

The PICO workflow

The PICO workflow comprises three simple steps: mixing of the lysed sample with oligonucleotide-labeled antibodies, formation of the antibody-protein complexes (termed 'couplexes'), and detection of couplexes using digital polymerase chain reaction (dPCR). The workflow is effectively counting target couplexes without loss and without background. For more information, refer to the PICO Handbook and Actome website. PICO provides different quantification possibilities: it can be used with an external reference as usual or directly compare two signals without a reference - relative quantification, and it can quantitatively measure the molar concentration of analytes without using an external reference - absolute quantification.

Measuring analytes without an external reference allows the analysis of samples where an appropriate external reference is not available, such as protein interactions, post-translational modifications or other complex molecular entities, or where the external reference may not accurately represent the sample composition. Absolute quantification delivers a more accurate assessment of the analyte's concentration in the sample by eliminating variability introduced by an external reference, making the assay less complex and often more cost-effective.

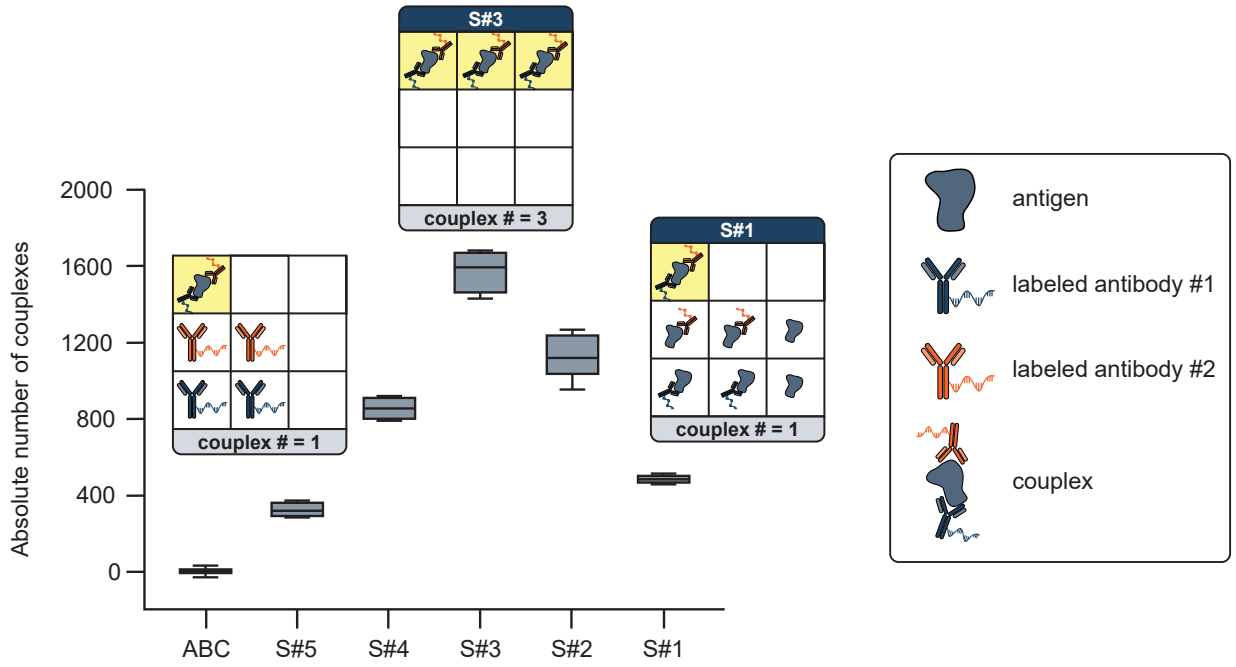

Figure 1. Typical result of measuring an antigen dilution series with PICO: a bell-shaped curve
Boxplots representing the absolute number of couplexes detected at different dilution steps (from highest 'S#5' to lowest 'S#1' dilution). The pattern of the boxplots resembles a bell-shaped curve. Ratios between antigen and labeled antibodies are depicted at three different dilution steps (S#5, S#3, and S#1). Couplexes are marked with a yellow background. Samples S#5 to S#3 represent the low side of the curve (LSC), while S#3 to S#1 represents the high side of the curve (HSC). The central solid line within each box is the mean, and the bottom and top of each box represent the 25th and 75th percentiles, respectively. The whiskers represent the 10th and 90th percentiles. ABC - antibody control.

Analytical behavior of the PICO assay - introduction to quantification

Performing a PICO assay with a dilution series of a biological sample generates a bell-shaped curve (theoretical calibration curve - TCC) when the couplex concentration is plotted against the dilution series of the antigen, assuming an adequately high starting antigen concentration (**Fig. 1**). On the low side of the curve (LSC), where the antibody concentration exceeds the antigen concentration, the number of formed couplexes is limited by the antigen availability. Increasing the antigen concentration on the LSC leads to a proportional increase in the number of couplexes. Within a specific range, a linear (on a log-log scale) relationship exists between the increasing antigen concentration and the resulting couplex formation. The equilibrium point represents the peak

of the curve, where a mathematically more complex relationship between antigen and couplex formation exists. However, as the antigen concentration surpasses the antibody concentration, the high side (HSC) of the curve is observed. In this region, the increasing antigen concentration competes for antibody binding, resulting in decreasing couplex concentrations. Within a specific range, a linear (on a log-log scale) relationship characterizes this relationship as well (**Fig. 1**).

PICO has a large dynamic range and high sensitivity (even average antibodies could set up an assay with a limit of detection - LOD - in the upper femtomolar range) and typically covers more than four magnitudes of concentrations. Typically at physiological target concentrations, PICO has measurements on the LSC, left of the equilibrium peak. However, there are cases where the HSC (right of the equilibrium peak) of the curve can be also seen with physiological targets.

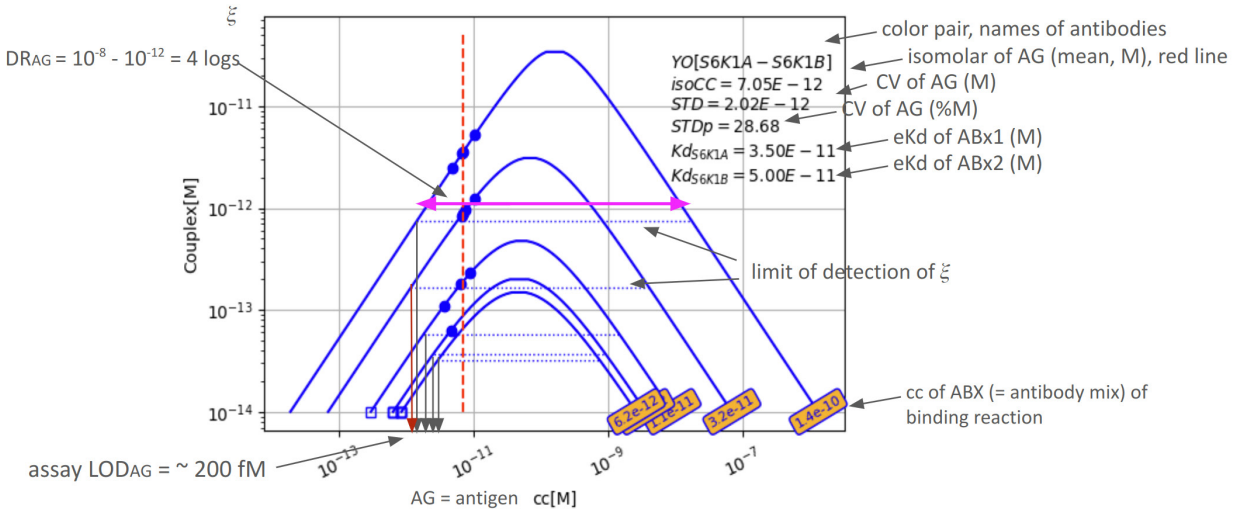

Figure 2. Theoretical calibration curves (TCCs) at different antibody concentrations.
The theoretical calibration curves (TCCs) are calculated according to the PICO theory, by using the molar concentration of antibodies (ABX) (indicated for each curve in orange squares) and the dissociation constants (K_D) of antibodies (one for each antibody). The x-axis shows the concentration of the antigen (ag), while the y-axis represents the concentration of couplexes (ξ - /ksai/), both represent molar concentrations in the binding reaction. Each curve has a dotted horizontal line indicating the lowest detectable amount of ξ (limit of ξ - Loξ) based on the lowest measurable dPCR counts of ξ. At the left end interception with the TCC of the Loξ line the ag readings are the LODs of the antigen detection (LODag - downward arrows). Note that LODag has an optimum around 3.2 x 10^{-11} of ABX (red downward arrow, roughly 200 fM in this example). DRag indicates the dynamic range of the measurement of antigen, and it is different for different ABXs (for the lowest LODag it is indicated as a pink arrow with an approximate).

The concentration of antibodies also impacts the TCC in PICO. Higher antibody concentrations lead to increase of the LSC, up to a saturation point. Beyond this saturation point, further increases in antibody concentration do not result in an increase in LSC. As shown in **Figure 2**, at saturation, the measured concentration of couplexes equals the concentration of the analyte. ***This observation, along with the absence of molecule loss and zero background in PICO, indicates that PICO is an absolute quantitative method under saturation conditions. In other words, PICO can measure the analyte concentration without the need for external standards, as the direct molar readings of couplexes exactly tell the molar concentration of the target analyte.***

Relative quantification (RQ) is a widely employed method that allows for the comparison of expression levels, offering valuable insights into quantitative changes in biological systems. RQ is closely connected to AQ in that they both leverage the linear log-log behavior exhibited by the TCCs. However, an important distinction is that RQ can be employed across a wide range of antibody concentrations, whereas AQ is only applicable when the

antibody concentration reaches a state of saturation. Also AQ primarily operates on the low side of the curve. RQ provides valuable information in the form of molar ratios, allowing for relative molar comparisons between expression levels. On the other hand, AQ provides measurements in molar units, offering absolute quantification of analyte concentrations.

How to use AQ?

For AQ, as discussed above, saturating antibody concentration is required. Usually, the K_D of the antibodies is unknown, thus it is recommended to set up a titration experiment using any antigen source, which could be a recombinant antigen or a cell lysate with a detectable amount of the specific antigen. In this experiment, the antigen concentration is kept constant while the concentration of the antibody mix is varied. This type of experiment is referred to as an isomolar titration (IMT), as illustrated in **Figure 2**.

It is noteworthy that increasing the antibody concentration causes the LSC to approach a saturation limit asymptotically, eventually reaching a specific concentration of the antibody mix, known as the saturation concentration. This effect means that the measured couplex concentration no longer increases with the increased concentration of the antibody mix. Notably, this effect occurs exclusively on the low side. Determining this saturation concentration is needed once for a given pair of antibodies and is applicable for all subsequent experiments. Typically, an antibody concentration of 5×10^{-10} M is suitable to achieve saturation for an average antibody, although it may vary. Using oversaturating concentrations does not pose any issues, but it unnecessarily reduces the assay's sensitivity due to the higher required dilution.

Once the saturation concentration is determined and used for the PICO assay, the analyte concentrations on the LSC are measured absolutely using the PICO method. This means that the determined concentrations of the couplexes correspond to the concentrations of the analytes. However, carrying out such AQ experiments necessitates additional considerations, which will be discussed below, along with an accompanying example. Additionally, it is crucial to emphasize that the peak region cannot be used for AQ or RQ analysis due to the loss of the linear correlation between the concentration of couplexes and the concentration of analyte within that particular region.

How to use RQ?

Using RQ is simple, regardless of the antibody concentrations employed, the ratio of the couplexes provides molar ratios. Unlike in the AQ method, there is no need for isomolar titration in RQ. Moreover, it is important to note, as in the case of AQ, that the peak region is not suitable for analysis, as the linear relationship between the concentration of couplexes and the concentration of analyte is no longer maintained in that region.

Materials and Methods

Trastuzumab (TTZ) and pertuzumab (PTZ), recombinant humanized monoclonal antibodies targeting extracellular regions of the HER2 tyrosine kinase receptor, were labeled with PICOglue BL or P8 Labels (#PICO-000120 & 21), respectively, using the PICOglue Antibody Labeling Kit (#PICO-000110). For the RQ analysis, one million BT474 and MCF7 cells were lysed according to the PICO Amplification Core (AMC) Kit (#PICO-000010) protocol. BT474 cells were first diluted 64-fold followed by a 1.5-fold dilution series (96-, 144-, 216- and 324-fold dilution samples). For the MCF7 cells an undiluted cell lysate followed by a 1.5-fold dilution series (1.5-, 2.25-, 3.375- and 5.0625-fold dilution samples) was used. For the AQ analysis, recombinant HER2 was analyzed employing a four-fold dilution series. For the binding reaction 2 µl of diluted sample was mixed with a total of 2 µl labeled PTZ and TTZ antibody and incubated overnight. The antibody concentration in the binding reaction for the RQ workflow was 4×10^{-11} M, while for the AQ analysis was 5×10^{-10} M. For the dPCR step, we aimed for an average lambda of 0.15, as recommended in the PICO AMC protocol. The dPCR was performed, using QIAGEN's QIAcuity Digital PCR System according to the PICO Amplification Core Kit protocol using the matching PICO BL and P8 Probes (#PICO-000070 & 71). The raw dPCR data was analyzed using Actome's AMULATOR software. The raw couplexes

were processed as described in the PICO Protein Detection Trial Kit manual, incorporating both ABC correction and labeling efficiency correction. ABC correction accounts for any offsets in the dPCR data, such as signal dropouts or incorrect clustering, while labeling efficiency correction adjusts for the actual number of formed couplexes. Applying the appropriate statistical test, it was assumed that the data followed a normal distribution based on the theoretical statistical distribution of couplexes. The calculation details can be found in the PICO Calculator.

Results

To demonstrate absolute quantification, refer to **Figure 3**. The recombinant antigen (HER2) was assessed through a four-fold dilution series, with concentrations of 4×10^{-11} M, 1.6×10^{-10} M, 6.4×10^{-10} M, and 2.56×10^{-9} M, respectively. These concentrations correspond to the PICO AQ measured absolute concentrations of $4.01 \times 10^{-11} \pm 1.02 \times 10^{-11}$ M, $1.59 \times 10^{-10} \pm 1.54 \times 10^{-11}$ M, $6.61 \times 10^{-10} \pm 4.45 \times 10^{-11}$ M, and $4.24 \times 10^{-9} \pm 3.10 \times 10^{-11}$ M, respectively. The BCA (bicinchoninic acid) method quantifies protein concentration by utilizing the biuret reaction, a chemical process that is not influenced by protein conformations. In contrast, PICO relies on antibodies and is sensitive to protein conformations, thereby accounting for the observed discrepancies between the two approaches. In a similar manner, the experiment allowed for the recovery of the four dilution ratios. The ratios of the sample from S#1 to S#4, as determined by AQ data, were found to be for S#1/S#2 6.12, S#2/S#3 3.9, and S#3/S#4 4.4, respectively. Conversely, when employing the RQ approach based on the ratios of the number of couplexes, the corresponding values were determined to be 0.6, 4.0, and 4.4. It is noteworthy that in the peak regions of the theoretical calibration curve (TCC), both methods exhibit some uncertainty due to the complex behavior of the TCC. Consequently, the RQ method is considered unreliable, as mentioned previously, while the AQ method remains reliable. However, a slight deviation from the intended dilution may still be observable.

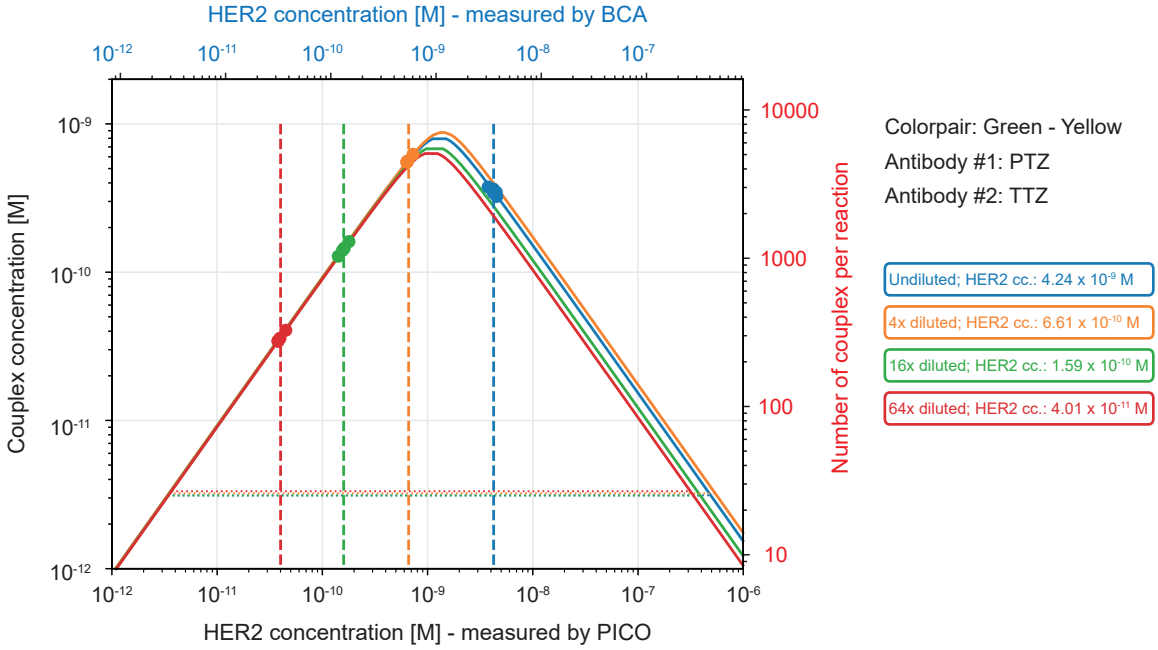

Figure 3. Absolute quantification of recombinant HER2 amounts
The left y-axis represents the measured couplex concentrations in the binding reaction in mol/l (M), while the right y-axis represents the measured number of couplexes in the dPCR reaction mix after ABC and labeling efficiency correction. The lower x-axis indicates the absolute concentration calculated using the PICO Calculator. The upper x-axis represents the concentration of recombinant HER2 proteins measured by BCA method. The curves refer to the mathematical model underlying the AQ theory, and thereby represent the correlation between the couplex, antigen and antibody concentration. The colors of the curves indicate the different antibody concentrations (given in the boxes on the right). The colored dots represent the measured samples. Vertical dashed lines represent the average antigen concentration of the sample, while the horizontal dashed line represents the LOD concentration. The boxes on the right give detailed information about the sample and measured antigen concentrations of the corresponding sample.

A similar relative quantification (RQ) experiment was conducted using a 64-fold diluted BT474 cell lysate sample compared to undiluted MCF7 cell lysate. The average fold change between the cell lines was determined to be 122.16 (**Fig. 4A**), indicating a significant difference in HER2 expression levels between BT474 and MCF7 cell lines. To depict the data as a volcano plot, the fold change values were transformed into log2 values and plotted against the negative logarithm (base 10) of the corresponding p-values (**Fig. 4B**).

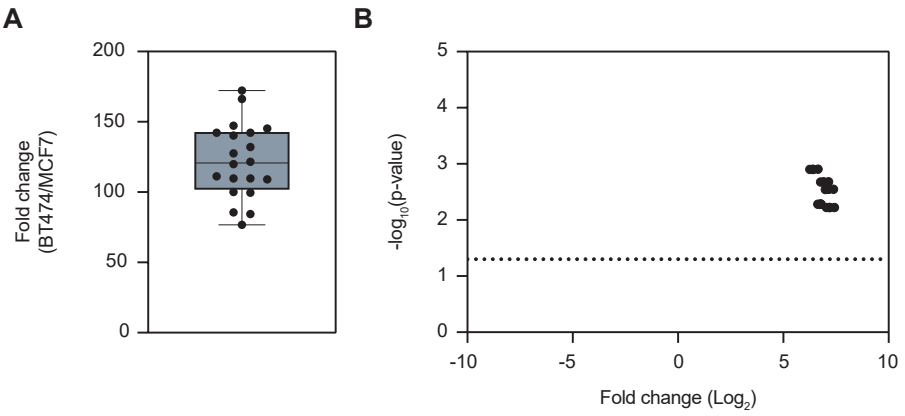

Figure 4. Relative quantification of HER2.
A) the normalized couplex counts of dilution samples from both cell lines (n=20) were used to relatively (RQ PICO) compare the amount of HER2 content of the cell lines. The resulting fold change values are depicted as a box plot. **B)** the transformed p-values and fold change values are visualized as a volcano-style plot. The dotted line indicates the threshold for statistical significance ($p < 0.05$) based on the transformed p-values.

Conclusion

In conclusion, the PICO technology combined with relative (RQ) and absolute (AQ) quantification provides a powerful and versatile approach for analyzing protein levels, such as HER2, in different cellular contexts. RQ allows for the comparison of expression levels, while AQ enables absolute quantification of analyte concentrations. The comparison of HER2 protein amounts in BT474 and MCF7 cells using PICO with RQ revealed significant differences in expression levels. This methodology offers valuable insights into protein quantification and can be applied to various research and clinical applications.

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