Quantification of Protein-Protein Interactions Using PICO Technology

Tobias Gross, Pablo Sánchez-Martín, Tamás Szórádi, Philipp Lübbert, and Csaba Jeney

Actome GmbH, Freiburg im Breisgau, Germany

Introduction

Cells are controlled by a complex network of signaling pathways that are constantly modulated in response to environmental stimuli. Dysregulation of these networks can lead to the onset of a wide range of human diseases like cancer (1). Thus, the understanding of how proteins interact with each other in signaling pathways is critical in biological sciences.

A well-studied example of protein-protein interactions (PPI) is found in the ERBB family of tyrosine kinase receptors (2). ERBB receptors are composed of a ligand-binding extracellular domain, a transmembrane domain, and a cytosolic domain with kinase activity. One of the ERBB receptors, ERBB2, is an orphan receptor, while ERBB3 lacks kinase activity (2). Together, the ERBB2:ERBB3 heterodimer regulates key downstream signaling pathways, which correlates with the aggressiveness and treatability of the tumor, as well as overall patient survival (2). To counteract ERBB2 hyperactivation in cancer, one of the main therapeutic approaches has been blocking its dimerization with ERBB3 through the use of the humanized antibody Pertuzumab (3).

Highlights

- Absolute quantification of protein-protein interactions (PPI) without external standard
- Easy multiplexing for detecting proteins and PPIs in parallel
- Confirmation of quantitative results by internal multiplexing
- Proteins and PPIs detected as number of proteoforms per cell
- PICO is ~300 times more sensitive than coIP
- Minimal input sample requirement (few 1000s cells)

Traditionally, PPIs have been most frequently studied by co-immunoprecipitation (coIP), a technique that requires high input material (1-3 mg of protein). In cases when the abundance of the target proteins is low, or the interaction is too transient, approaches like overexpression need to be applied, which in turn could falsify the physiological behavior of the proteins, leading to erroneous results. For example overexpressed proteins might exhibit interactions not present at endogenous levels (4). After coIP, the samples are commonly analyzed by western blot, a method whose limitations have been extensively discussed elsewhere (4), including limited quantification precision and sensitivity. In this application note we used the ERBB2:ERBB3 interaction as a model to develop a highly sensitive and absolutely quantitative Protein Interaction Coupling (PICO) assay.

Results

First, we confirmed the endogenous ERBB2:ERBB3 interaction using coIP in BT474 cells, a breast cancer cell line overexpressing ERBB2 (**Figure 1**). In contrast, in MCF7 cells, a HER2 '*negative*' cell line, the interaction could not be detected (data not shown). We attributed this to the limited sensitivity of the coIP and western blot, and thus we aimed to test the ERBB2:ERBB3 interaction using PICO, a highly sensitive and absolute quantitative digital immunoassay.

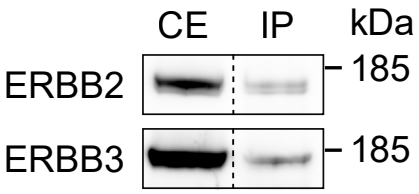

Figure 1. Co-immunoprecipitation of ERBB2:ERBB3 interaction in BT474 cells. Samples, corresponding to 800,000 cells, were immunoprecipitated overnight with anti-ERBB3 antibody. After washing, the captured proteins were analyzed by western blot using ERBB2 and ERBB3 antibodies as indicated. CE: crude extract; IP: immunoprecipitation.

We designed a quadratic (using four antibodies) PICO assay which quantifies both ERBB2 and ERBB3 proteins separately, in addition to their interaction (**Figure 2**). Using as little as 5,000 BT474 cells (2.5 µg of cell lysate) we could confirm the interaction (**Figure 3A**). In comparison, the coIP experiment required 800,000 cells. This corresponds to an at least 300 fold sensitivity gain over coIP. Moreover PICO enables absolute quantification as well (5), allowing the measurement of '*proteoform copy numbers per cell*' [ppc]. According to the absolute quantification, the expression of ERBB2 (957,618 ± 29,339 ppc) was 738 times higher than the expression of ERBB3 (1,297 ± 166 ppc) in BT474 cells, as expected from previous publications (6).The ppc difference between ERBB3 protein and ERBB2:ERBB3 interaction, was not statistically significant, indicating that essentially all copies of ERBB3 are in a complex with ERBB2 in the BT474 cell line, while only a 0.11% of the total ERBB2 amount is interacting with ERBB3.

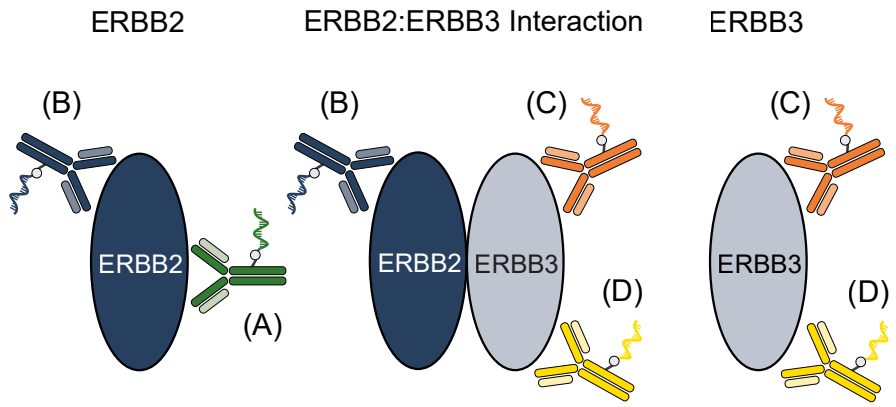

Figure 2. Detection strategy for ERBB2, ERBB3, and ERBB2:ERBB3 interaction by PICO. Two non-overlapping monoclonal antibodies were selected for each of the target proteins (ERBB2 - A and B, ERBB3 - C and D). The PICO readout is based on digital detection of couplexes, the ternary complexes of two antibodies and the proteoform. This setup allows the simultaneous detection of ERBB2 (A-B antibody pair) and ERBB3 (C-D antibody pair) total protein amount, and their interaction (combination of one antibody for ERBB2 and one antibody for ERBB3, e.g. B-C or B-D antibody pair).

One of PICO's inherent features is 'confirmatory multiplexing', which is based on the ability to readout any combination of antibodies (see also our application note about quantification of the phosphorylation status of 4EBP1) (5,7)). Using readings of two independent antibody pairs (B-C and B-D antibody pairs, see **Figure 2**) resulted in quantifying the same amount of ERBB2:ERBB3 interaction, confirming the copy-number of the ERBB2:ERBB3 protein interaction (**Figure 3A**).

To further challenge the sensitivity of the PICO assay, we carried out the same assay as above using MCF7 cells, an ERBB2 '*negative*' breast cancer cell line. In agreement with previous reports (6), ERBB2 expression confirmed its '*negative*' low-expressing status (10,938 ± 509 ppc), which is 87 times less compared to BT474, while the ERBB3 expression was (3,666 ± 481 ppc), which is 1.41 times higher than in BT474 cells. The ratio of ERBB2 to ERBB3 expression is 738 in BT474 cells, while in MCF7 cells it is only 2.98. These results were achieved using 20,000 cells (10 µg of cell lysate) underlining the high assay sensitivity once again (**Figure 3B**).

We also aimed to quantify the effects of drug treatments by using Dactolisib/BEZ235, a dual PI3K and mTOR inhibitor that induces the interaction between ERBB2 and ERBB3 (8). Previously, the effect of Dactolisib on ERBB2:ERBB3 interaction had only been observed upon long treatments (8), which in our hands resulted in decreased cell survival likely arising from the effect of the drug on protein translation (unpublished data). We reasoned that

protein interaction changes can occur earlier, and thus 4 h treatment with 5.6 µM Dactolisib was used, measuring a 2.32 fold increase in ERBB2:ERBB3 interaction in MCF7 cells, which is in line with published data (**Figure 3C**).

Conclusion

CoIP and western blot have a set of limitations (4). To overcome these limitations, PICO was applied to study the ERBB2:ERBB3 interaction in both BT474 and MCF7 cells. Absolute quantification of the data showed a much higher ERBB2 to ERBB3 ratio in BT474 cells than in MCF7, confirming a high level of free ERBB2 protein in BT474 cells compared to MCF7 cells. Combined with the interaction data, we could estimate that all ERBB3 is in interaction with ERBB2 in BT474 and in MCF7, and in MCF7 cells the interaction can be modulated by drug treatment. Based on these results, we demonstrated that PICO provides an absolute and sensitive quantification approach to measure proteins and their interactions, with low input cell requirement, and with robustness. In addition, we quantified multiple ways the same protein, internally confirmed quantitative results with a sensitivity down to a few hundred of proteoforms per cell, and demonstrated that PICO is at least ~300 times more sensitive than coIP. We are confident that PICO can be applied to quantify perturbations of proteoforms and protein-protein interactions even of clinical relevance.

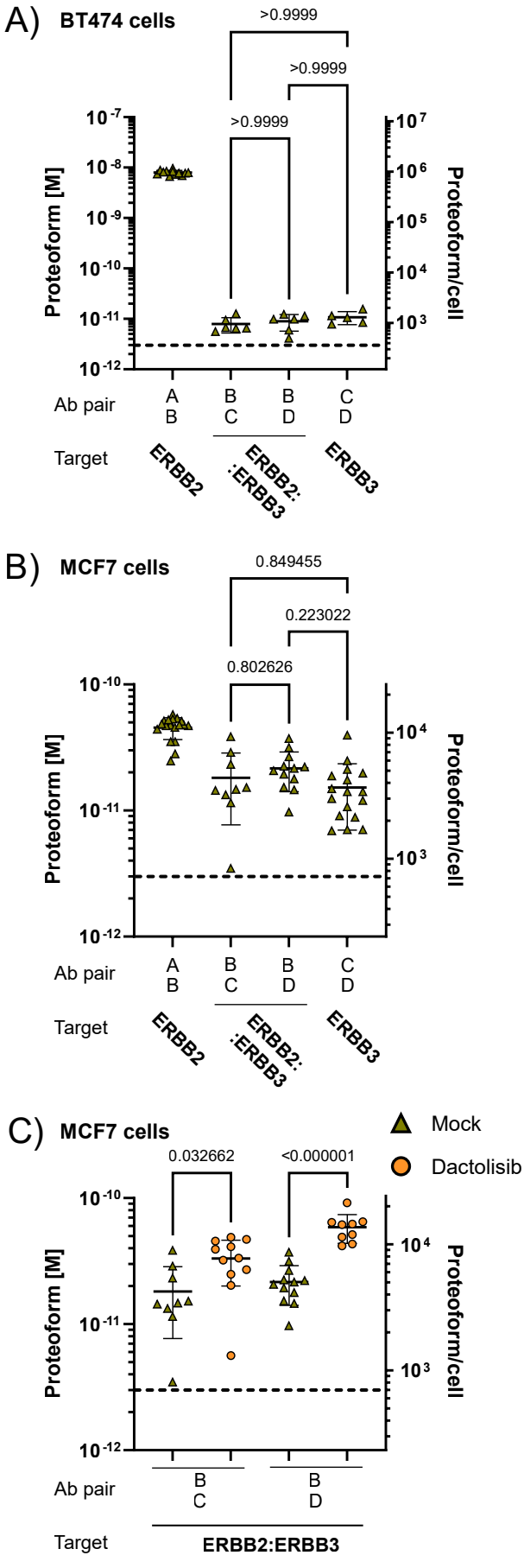

Figure 3. Detection of ERBB2, ERBB3, and ERBB2:ERBB3 interaction in BT474 and MCF7 cells. A) Absolute quantitative results of BT474 cells down to 5,000 cells (dilution compensated results) are represented as total molar concentration (left Y axis, molar concentration in the binding reaction) or number of proteoform copies per cell (right Y axis). **B)** Absolute quantitative results of MCF7 cells down to 20,000 cells (dilution compensated results) are represented as total molar concentration (left Y axis, molar concentration in the binding reaction) or number of protein copies per cell (right Y axis). **C)** Comparison of ERBB2:ERBB3 interaction in MCF7 cells treated for 4 h with DMSO (Mock) or 5.6 µM Dactolisib. Two different antibody pairs (B-C and B-D, Figure 2) were compared to simultaneously detect ERBB2:ERBB3 interaction. Dashed line: limit of detection.

Materials and Methods

PICO assay

Monoclonal antibodies targeting non-overlapping epitopes on the extracellular domains of ERBB2 and ERBB3 were labeled with PICOglue Labels (#PICO-000120 to 123) sing the PICOglue Antibody Labeling Kit (#PICO-000110). The indicated amounts of BT474 and MCF7 cells were lysed according to the PICO Amplification Core Kit (#PICO-000010) User Manual. For the binding reaction, 2 µl of cell lysate was mixed with a total of 2 µl labeled antibodies at a concentration of 500 pM and incubated overnight. For the dPCR step, we aimed for an average lambda of 0.15, as recommended in the PICO Amplification Core Kit protocol. The dPCR was performed using QIAGEN's QIAcuity Digital PCR System using the matching PICO Probes (#PICO-000070 to 73). 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 User Manual, incorporating both ABC compensation and labeling efficiency correction. ABC compensation accounts for any offsets in the dPCR data, such as signal dropouts or incorrect clustering, while labeling efficiency correction adjusts for the 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 (5).

Co-immunoprecipitation (coIP)

For the coIP analysis, the cells were lysed according to the PICO Amplification Core Kit User Manual. ERBB2:ERBB3 complexes were captured overnight using anti-ERBB3 antibody and Protein A/G Agarose (Thermo Fisher). After three washing steps, the samples were eluted from the beads by incubation at 95°C with 1x NuPage LDS Sample Buffer (Thermo Fisher). Then, the samples were loaded onto NuPAGE Novex 4-12% Bis-Tris Gels (Thermo Fisher). Blotting was carried out with the iBlot 2 GelTransfer Device. Probing the membrane with the antibodies and the washing steps were performed using the iBindFlex Western Kit (both Thermo Fisher). For detection, the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was used with an ImageQuant 800 Western blot imaging system (Amersham).

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Actome GmbH • Georges-Köhler-Allee 302 • 79110 Freiburg im Breisgau, Germany • Tel. +49 761 216 305 00

actome.de info@actome.de @actomegmbh linkedin.com/company/actome @actome.gmbh youtube.com/@actome