Highly sensitive HER2 detection in BT474 and MCF7 cells using the PICO technology

Tobias Gross, Paula Dewes, Tamás Szórádi, Philipp Lübbert and Csaba Jeney

Actome GmbH, Freiburg im Breisgau, Germany

Introduction

Breast cancer is one of the most frequent cancer types and causes the majority of cancer-related deaths in women around the globe (1). Breast cancer is classified into different subtypes. One subtype, occurring in 14% to 29% of cases, is the HER2-positive breast cancer (2). These cancer cells overexpress the protein ERBB2 (also known as HER2), a member of the human epidermal growth factor receptor family. Genomic amplification or overexpression of this oncogene plays an important role in the development of HER2-positive breast cancer and is the major factor behind the augmented proliferation of this aggressive breast cancer type. Furthermore, HER2-positive breast cancer has a preference for brain metastasis (3). Anti-HER2 cancer immunotherapy is the most frequently used therapeutic intervention to treat the disease using therapeutic antibodies,such as trastuzumab or pertuzumab. Recently, the combination therapy with these antibodies has been approved by FDA for treatment of breast cancer as neoadjuvant therapy (to shrink the tumor before surgery) in adults and as adjuvant therapy in adults with early-stage breast cancer without prior testing for HER2 positivity. These new indications are based on the combination blockage of the not-augmented, normal HER2 signaling and hence reducing tumor growth. Thus HER2 testing can be of high importance for biomedical research. Methods which are capable of detecting the extremely low level of HER2 expression in cell based in-vitro disease models could be of high value in this context.

Highlights

- Comparing the limit of detection (LOD) of western blot and PICO (Protein Interaction Coupling), using the established BT474 and MCF7 breast cancer cell lines.
- PICO shows higher dynamic range and lower LOD compared to western blot.
- High sensitivity protein detection down to femtomolar level.

In research, immortalized breast cancer cell lines are widely used to investigate the underlying mechanisms of cancer development and to develop new cancer therapies. Two of the most commonly used breast cancer cell lines are the HER2-positive BT474 and the HER2-negative MCF7 cell line (4). While HER2 is overexpressed in BT474 cells (approximately 150,000

copies per cell, unpublished data), MCF7 cells show a much lower abundance of HER2 (approximately 500 copies per cell) (5). High level of HER2 (e.g. in BT474 cells) is easily detectable with common laboratory methods like western blotting. However, detection of HER2 at low expression levels is a challenge with such methods. Therefore, cell lines might be considered HER2-negative, while they are still expressing HER2 at low levels.

Actome's Protein Interaction Coupling (PICO) technology is designed for ultra high sensitivity protein detection. The main features of PICO, including the femtomolar sensitivity and low input sample volume (down to one microliter). This typically results in a LOD of 10,000 to 100,000 proteins, depending on the antibodies used for detection. PICO combines the highly specific approach of an immunoassay using a pair of antibodies with the ultimately sensitive digital polymerase chain reaction (digital PCR; dPCR) for detection of the antibody pair. The PICO workflow follows a three step procedure: mixing sample and the antibodies, incubating the mixture to achieve binding between the antibodies and the target, and finally detecting the formed molecular complexes in dPCR. For simplicity, the complex of two bound antibodies to the target protein is called 'couplex' (**Fig. 1**). The antibodies are labeled with specific oligonucleotide labels, the PICO Labels. For the PICO technology to work, two antibodies, recognizing different epitopes of the target protein are needed. During the dPCR reaction, the PICO Labels are amplified and together with specific PICO Probes fluorescent signals are generated. The fluorescent signals arising from individual antibody molecules are detected by the dPCR instrument. Further details about the PICO technology can be found in the **PICO Handbook** and on the Actome **website**.

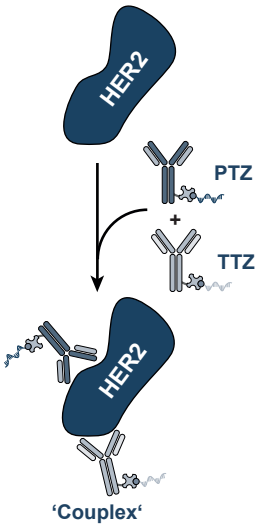

Figure 1. The detection unit of the PICO assay is termed 'couplex'. Two differently labeled antibodies, here trastuzumab (TTZ) and pertuzumab (PTZ), bound to a single HER2 protein represent a couplex.

Here, we describe the PICO technology as a method that allows for the detection of HER2 in cell lines like MCF7, that are generally considered HER2-negative, but still express the protein in very low amounts.

Materials and Methods

Trastuzumab and pertuzumab are recombinant humanized monoclonal antibodies, both targeting extracellular regions of the HER2 tyrosine kinase receptor. Since they target different epitopes of HER2, they are suitable for the PICO assay (6). The PICO workflow (**Fig. 2**) was applied to label the antibodies and to detect HER2 in BT474 and MCF7 cell line. Both antibodies were conjugated with Actomidin using the PICOact Antibody Conjugation Kit protocol (#PICO-000030). The conjugated antibodies were labeled with two different labels (PICOact BL and PICOact P8 Label; #PICO-000060 & 61) following the PICOact Conjugated Antibody Label Loading Kit (#PICO-000040) protocol. Cell lysate from approximately 1 million cells was made according to the PICO Amplification Core Kit (#PICO-000010) protocol. The samples were diluted, combined with the antibodies, and were mixed with the PICO mastermix containing the PICO Probes (BL & P8; #PICO-000070 & 71). QIAGEN's QIAcuity Probe Mastermix was added and the samples were loaded onto a QIAcuity Nanoplate 26k 24-well plate. Digital PCR was performed using a QIAGEN QIAcuity Digital PCR System with cycling parameters according to the PICO Amplification Core Kit protocol. The raw dPCR data was analyzed using Actome's AMULATOR software.

For western blot analysis, the cells were lysed according to the PICO AMC workflow without cross-linking. The sample was mixed with NuPage LDS Sample Buffer 4x (Thermo Fisher) and the stated amounts of cells were

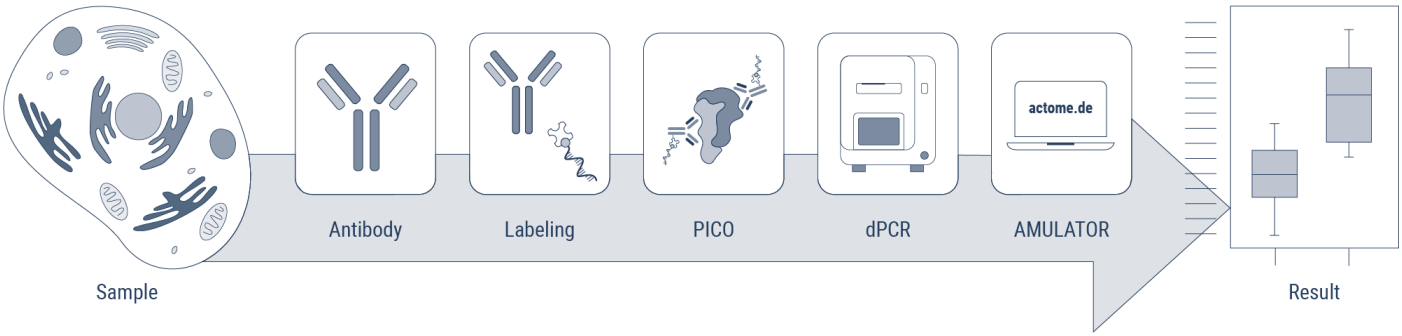

Figure 2. The PICO workflow starting from sample and antibody selection, antibody labeling to protein detection via PICO assay, with subsequent data analysis with AMULATOR.

loaded onto NuPAGE Novex 4-12% Bis-Tris Gels (Thermo Fisher). Blotting was carried out with the iBlot 2 Gel Transfer Device. Probing the membrane with the antibodies and the washing steps were performed using the iBind Flex Western Kit (both Thermo Fisher). Trastuzumab (TTZ) and GAPDH primary antibodies were used together with anti-human and anti-mouse secondary antibodies, respectively. For detection the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was used with an ImageQuant 800 Western blot imaging system (Amersham).

Results

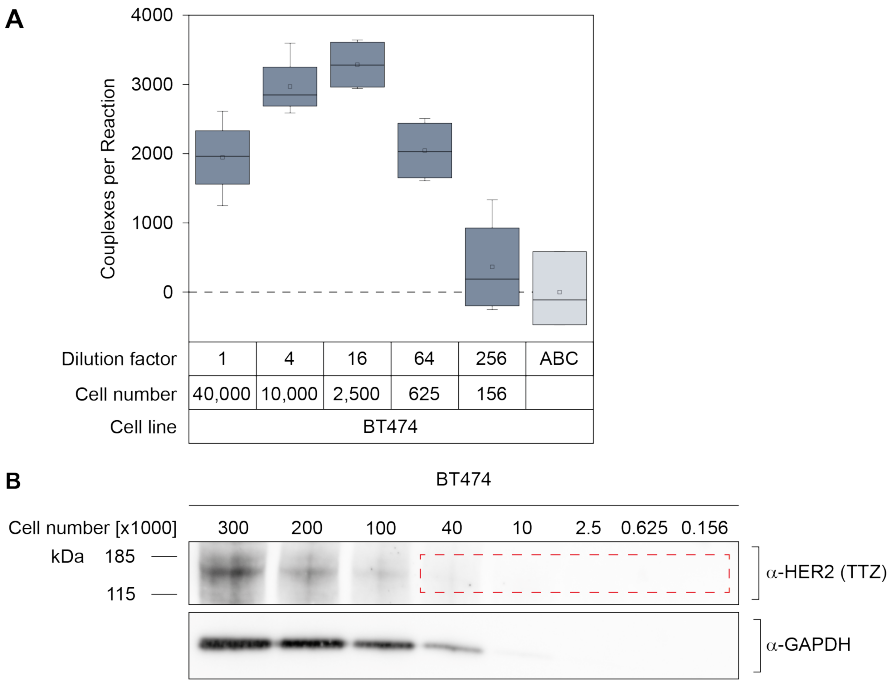

Figure 3. HER2 detection in BT474 cell line. **A)** Couplexes per reaction from different amount of BT474 cells that were detected in the PICO assay, using the HER2-specific antibodies PTZ and TTZ. **B)** Western blot of whole cell lysate dilution series of BT474 cells, probing done with HER2-specific antibody TTZ and GAPDH-specific antibody. The red box reflects the cell amounts that were used in the PICO assay.

First, we tested the sensitivity of PICO on BT474 cells, a cell line that is known to be HER2-positive. We labeled trastuzumab and pertuzumab with the PICO DNA Labels and the PICO assay was performed using these antibodies with BT474 cell lysates. A control sample, containing only PICO-labeled antibodies without a target present (termed ABC - AntiBody Control), was included. In the ABC sample, the detected number of couplexes must be close to zero, which proves that the antibodies are not interacting without the target protein being present.

We detected couplexes (HER2 bound by the two differently labeled antibodies) in the dilution series of BT474 cell lysate (**Fig. 3A**). The range of detected couplexes were between 3,500 and ~200, dependent on the dilution factor, and therefore the amount of cells used. The couplex count in the sample with the dilution factor of 256 (reflecting 156 cells), reached the ABC levels. This result suggests that the LOD is approximately at 200-fold

dilution, reflecting 200 cells. Next, we tested whether we can detect HER2 in BT474 cells with western blot, using the very same dilution series and cell numbers. As seen in **Figure 3B**, we could only detect HER2 from 300,000 to 100,000 cells with western blot. HER2 was barely visible in the sample containing 40,000 cells (the first dilution step in the PICO experiment) and was undetectable in the samples with lower cell count. This proves that the PICO assay was approximately 100,000/156 = 640 times more sensitive for HER2 protein detection than western blot with the same antibodies.

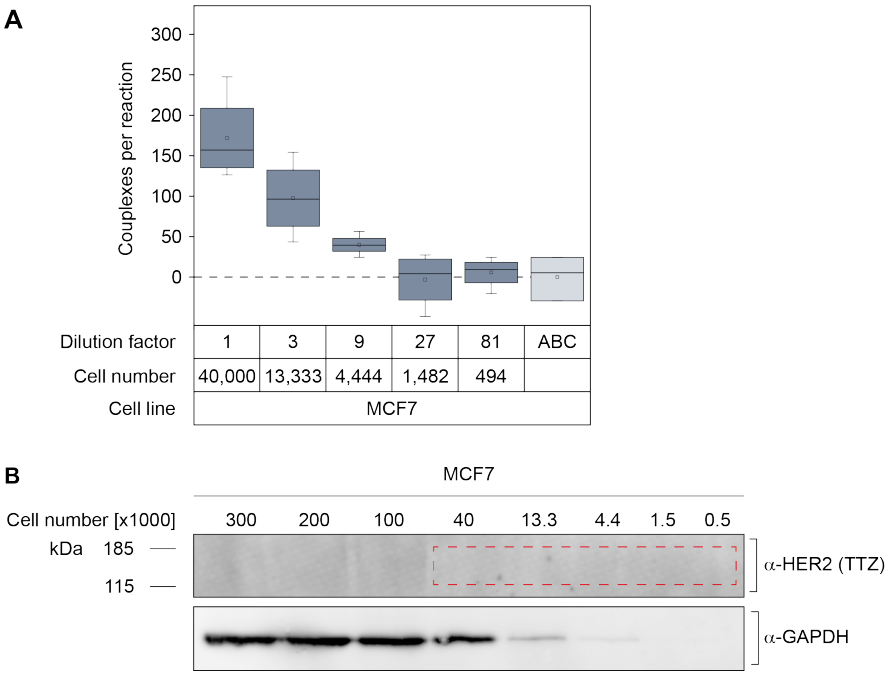

Figure 4. HER2 detection in MCF7 cell line. **A)** Couplexes per reaction from different amount of MCF7 cells that were detected in the PICO assay, using the HER2-specific antibodies PTZ and TTZ. **B)** Western blot of whole cell lysate dilution series of MCF7 cells, probing done with HER2-specific antibody (TTZ) and GAPDH-specific antibody. The red box reflects the cell amounts that were used in the PICO assay.

Next, we tested the limits of PICO sensitivity by detecting HER2 in MCF7 cells, a cell line commonly considered to be HER2-negative. We were able to detect HER2 proteins in MCF7 cells with PICO even at high lysate dilutions (**Fig. 4A**). The range of detected couplexes was between 175 and 50, depending on the dilution factor. The sample lysate from only 4,444 cells gave rise to a couplex count of approximately 50, and based on the ABC value, this can be considered to be close to the LOD of the assay. Furthermore, higher dilutions of MCF7 cell lysate (dilution factor 27 and 81) led consistently to zero couplex count, which also confirms the zero-background feature of the PICO technology. If proteins are not detectable either due to the high sample dilution or to their absence from the sample, no couplexes are detected and thus there is no background noise. Next, we attempted to detect HER2 in MCF7 cells using western blot. HER2 was undetectable even from samples containing 300,000 cells (**Fig. 4B**) or using as long as 30 minutes of exposure time. This result, is consistent with the measurements on BT474 cells above and proves that PICO can detect low levels of HER2 in MCF7 cells, despite the fact that this cell line is commonly considered to be HER2 negative.

Conclusion

The PICO technology enables the detection of ultra low amounts of protein in biological samples. The high sensitivity not only allows for detection of low abundant proteins, but also to use smaller sample volumes. Here we demonstrate that the PICO technology is able to quantify the amount of HER2 protein from only 4,444 cells using the MCF7 cell line that is commonly considered to be HER2-negative (compare for example (7), where they studied the same cell lines with western blot technology, using other antibodies). Diluting the cell lysate further results in zero signals, because the couplexes were diluted out. This impressively reflects the fact that there is no recognizable background noise present in PICO assays.

The ultra high sensitivity of PICO technology is a new tool that enables researchers to analyze low abundant proteins that were previously undetectable with other commonly established methods. The combination of the homogeneous immunoassay with dPCR technology based on single molecule detection, results in an extremely low limit of detection. A further benefit of high sensitivity is that it becomes possible to conduct experiments in a more time-saving way and with less financial effort, because total sample amount can be reduced.

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

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

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