

# splitPICO: a novel PICO workflow for multiplex and absolute quantitative analysis of entire signaling pathways

Tobias Gross, Christoph Niemöller, Pablo Sánchez-Martín, Pratika Agarwal, Marius Pollet, Tamás Szórádi, Nina Gross, and Csaba Jeney

Actome GmbH, Freiburg im Breisgau, Germany

## Introduction

Alterations in critical signaling pathways that regulate cell cycle progression, apoptosis, and cell growth are hallmark features of cancer. The frequency and mechanisms of these changes vary significantly across tumors and cancer types. In a groundbreaking study, researchers mapped oncogenic signaling pathways using data from The Cancer Genome Atlas (TCGA), analyzing 9,125 tumors across 33 cancer types and 64 subtypes. This comprehensive analysis investigated somatic changes in 10 pivotal pathways: cell cycle, Hippo, Myc, Notch, NRF2, PI-3-Kinase/Akt, RTK-RAS, TGF $\beta$  signaling, P53, and  $\beta$ -catenin/WNT. The findings revealed that 89% of tumors had at least one driver alteration in these pathways, and 57% harbored changes that could be targeted by currently available therapies. Even more compelling, 30% of tumors contained multiple targetable alterations, opening the door to promising combination treatment strategies (1). These insights offer exciting new avenues for precision medicine, targeting the molecular drivers of cancer with unprecedented accuracy and effectiveness.

However, quantitative analysis of entire signaling pathways is a challenge. To overcome the challenge we developed splitPICO, a novel workflow for the Protein Interaction Coupling (PICO) technology that allows parallel detection and quantification of up to 12 targets (proteins, protein-protein interactions, post translational modifications), enabling the proteomic investigation of signaling pathways.

We demonstrate the capabilities of splitPICO using the HER2 pathway, a key driver of tumorigenesis in breast cancer. HER2 and HER3 (Human Epidermal Growth Factor Receptor 2 and 3) are key proteins involved in the regulation of cell proliferation and division. The interaction between these proteins is a pivotal molecular event that triggers a signaling cascade, leading to the expression of genes that drive tumor growth and metastasis. This cascade primarily involves two pathways: the RAS/RAF/MEK/ERK pathway, which promotes cell proliferation and survival, and the PI3K/AKT/mTOR pathway, crucial for cell growth and metabolic regulation. Downstream of mTOR, key effectors such as the translation regulator 4EBP1 and the ribosomal kinase S6K1 modulate protein synthesis through cap-dependent translation (**Figure 1**). Overexpression of HER2 is strongly associated with aggressive cancer phenotypes, and the level of HER2 expression correlates with tumor aggressiveness, treatment response, and overall patient survival. However, it is the interactions and downstream signaling cascades, not merely the

## Highlights of splitPICO

- **Superior to traditional multiplex western blot** - a more efficient, sensitive, and quantitative way to analyze proteins, protein interactions, and post-translational modifications in a single experiment
- **Multiplex and absolute quantification** - simultaneous detection and absolute quantification of up to 12 targets
- **Easy workflow** - simple workflow with minimal hands-on time for high-throughput studies

presence of HER2, that are exploited by cancer cells (2). Therefore, studying all relevant molecular targets in parallel within a single experiment is crucial for understanding the complexity of the pathway and its response to therapeutic interventions.

## Results

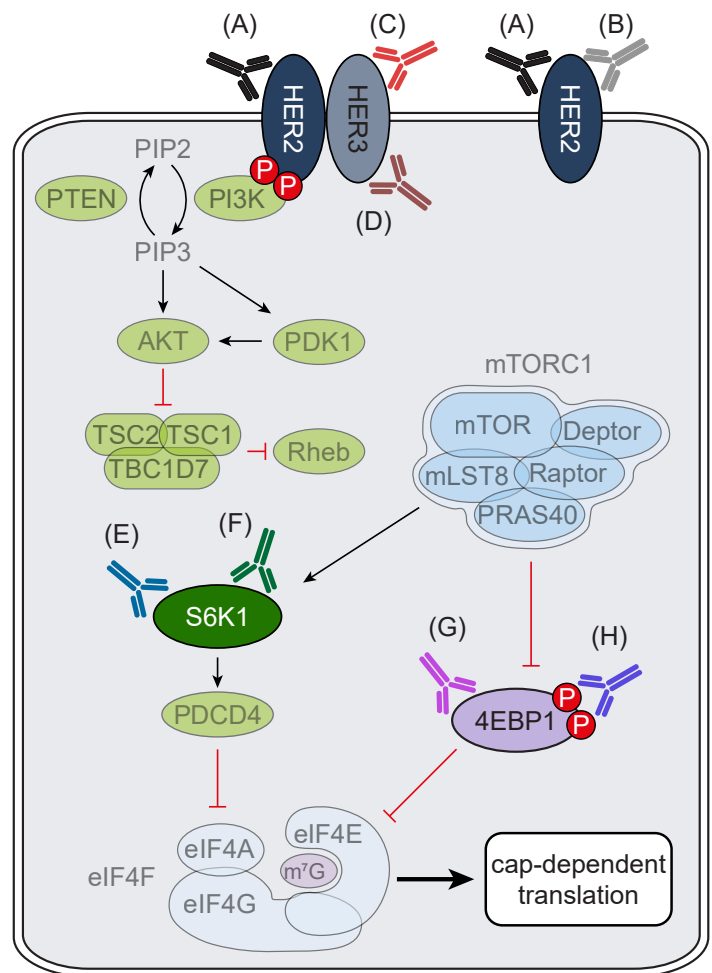
Conventionally in PICO each antibody is detected through a distinct fluorescent channel, however when the number of targets exceeds the available detection channels, additional PICO reactions are required. This increases the amount of sample needed, which could be a problem if the amount of the sample is a concern, introducing biological variability, and increasing the workload. With the innovative splitPICO workflow, we have significantly expanded the detection capacity, enabling the analysis of twice (or more) as many targets without additional sample requirements. This is achieved by introducing a new set of labels (PICOglue Beta Labels), which have distinct primer binding sites compared to the 'original' labels (PICOglue Alpha Labels) but the same PICO Probe binding site (**Figure 2A**). Consequently, the same PICO Probes can be utilized during the amplification of both Alpha and Beta Labels.

In the splitPICO workflow, the sample is first mixed and incubated with a panel of up to eight labeled antibodies: four antibodies labeled with distinct Alpha Labels and four with distinct Beta Labels. After incubation, the mixture is split into two master mixes (hence the name), one containing primers specific for the Alpha Labels and the other for the Beta Labels. During amplification the Alpha Primers will allow only the amplification of Alpha Labels while Beta Primers allow the amplification of Beta Labels (**Figure 2B**). This approach enables the parallel and multiplex analysis of multiple protein targets from a single reaction.

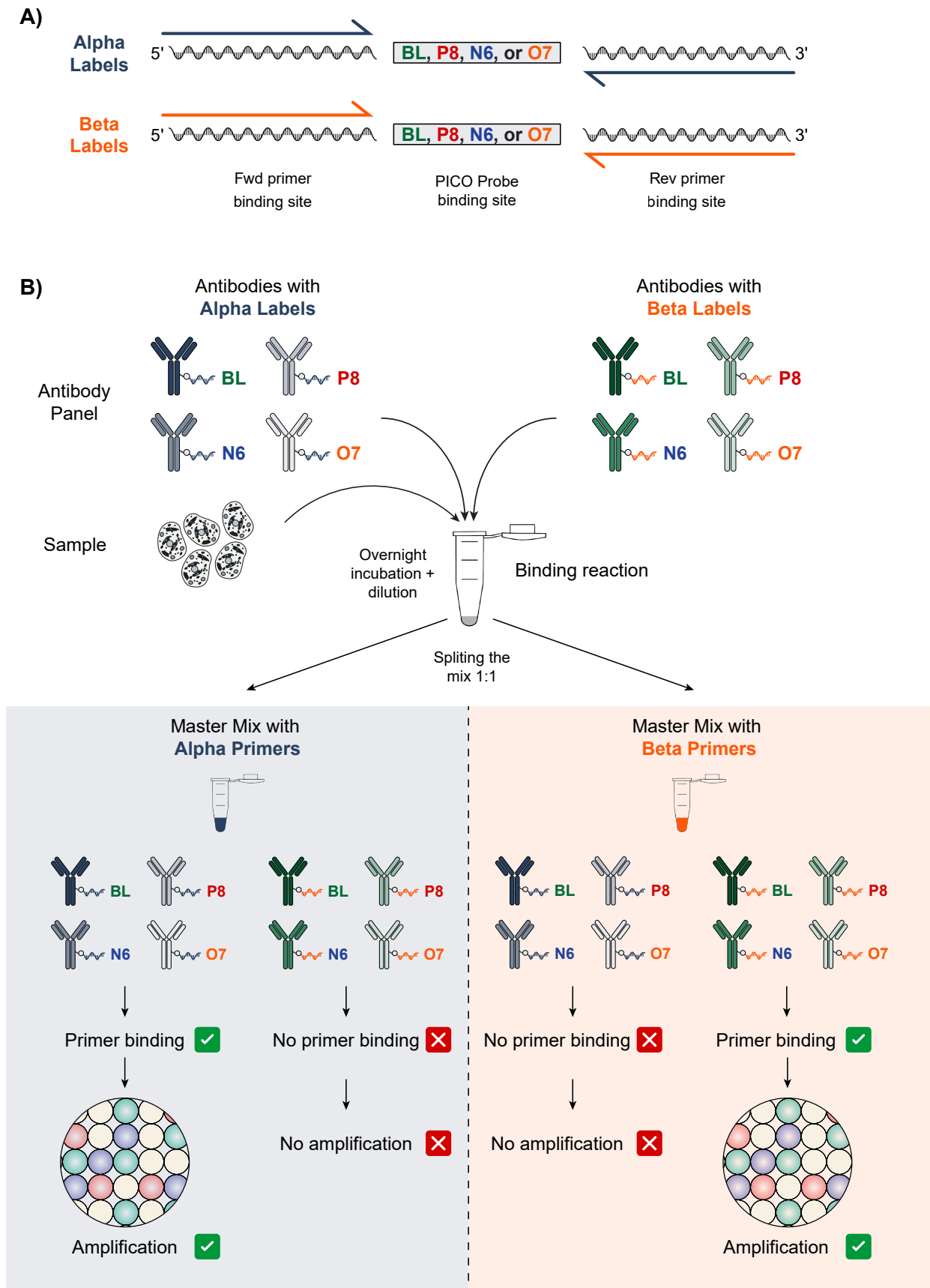
First we verified that the modifications in the oligonucleotide labels did not impact their amplification efficiency or target detection. Recombinant HER2 was simultaneously incubated with equal concentrations of HER2 antibodies labeled with either PICOglue Alpha or Beta Labels, and the samples were processed using the splitPICO workflow as outlined above. Both Alpha and Beta Labels yielded identical quantification results, thereby validating the robustness and reliability of the approach (**Figure 3**).

The splitPICO workflow was then applied to the five selected targets in the HER2 pathway: a cytosolic protein (S6K1), two membrane proteins (HER2 and HER3), a phosphorylated cytosolic protein (p4EBP1), and a protein-protein interaction (HER2::HER3 - measured with two different antibody pairs), resulting in a total of six detections. The eight necessary antibodies were used simultaneously but detected in separate amplification reactions: S6K1 and p4EBP1 in the Alpha reaction, and HER2 and HER3 in the Beta reaction.

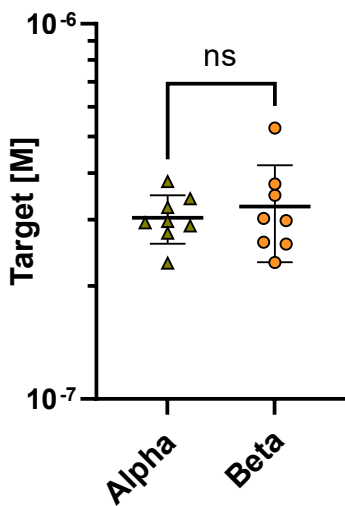
We successfully detected a significant number of complexes for all targets in both the Alpha (**Figure 4A**) and the Beta reaction (**Figure 4B**). The use of a large panel of antibodies not only allows for the detection of the original six targets but also incorporates built-in negative controls for the PICO assay. Antibody pairs



**Figure 1: Full pathway analysis using splitPICO.** Depiction of the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR signaling intersection downstream of HER2. The proteins included in the splitPICO experiments (HER2, HER3, S6K1, p4EBP1) are highlighted, and the abbreviations for the antibodies are indicated in parentheses.



**Figure 2: The splitPICO workflow. A)** The PICOglue Labels (Alpha and Beta) contain identical probe binding sites (P8, BL, N6, or O7) but differ in their primer binding sites. This allows the selective amplification of the label only when their specific primers (Alpha or Beta Primers) are present. **B)** Four antibodies conjugated with Alpha Labels and four conjugated with Beta Labels are combined with the sample in the binding reaction. After dilution, half of the binding reaction is transferred to a master mix containing the Alpha Primers, while the other half is transferred to a master mix containing Beta Primers. During amplification, based on the primers, different proteins from the same binding reaction will be amplified and quantified.

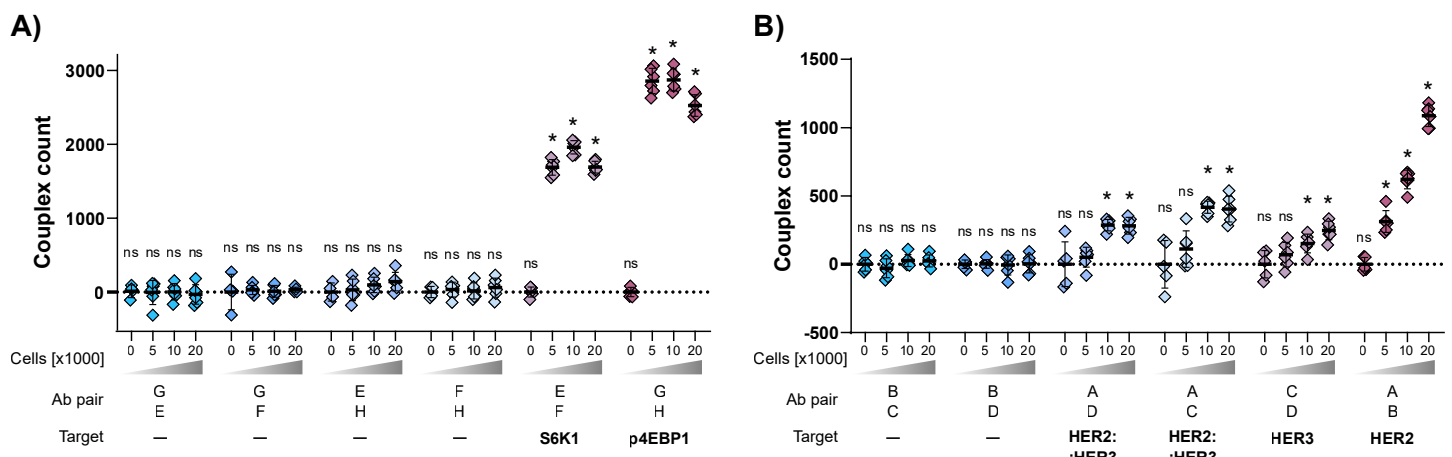


**Figure 3: splitPICO amplification control.** Alpha- and Beta-labeled antibodies can be equally used in PICO.

from irrelevant combinations, such as those binding to S6K1 and 4EBP1 (two proteins that do not interact), serve as validation controls, as they result in no detectable complexes (**Figure 4A**, antibody pairs of GE, GF, EH, and FH, and **Figure 4B**, pairs of AC, AD). The non-sense antibody pairs further confirm the precision of the identified interactions and contribute to the overall validity of the detections.

To maximize the potential of the splitPICO workflow, we applied it to investigate the effects of Dactolisib, a phosphorylation inhibitor of PI3K and mTOR, in two breast cancer cell lines that primarily differ in their HER2 expression levels (MCF7 and BT474). Here we used absolute quantification to gain insight into the stoichiometry of the proteins comprising the HER2 pathway and how their abundance and interactions are modulated in response to Dactolisib in a cell-specific manner. The mechanism of action of Dactolisib in the HER2 pathway is thought to occur through inhibition of mTOR and PI3K, which we confirmed by observing reduced 4EBP1 phosphorylation levels (**Figure 5A**) (3). In addition, the high sensitivity of PICO also detected smaller but significant changes in the protein levels of S6K1, HER2, and the HER2::HER3 interaction (**Figure 5B**), confirming

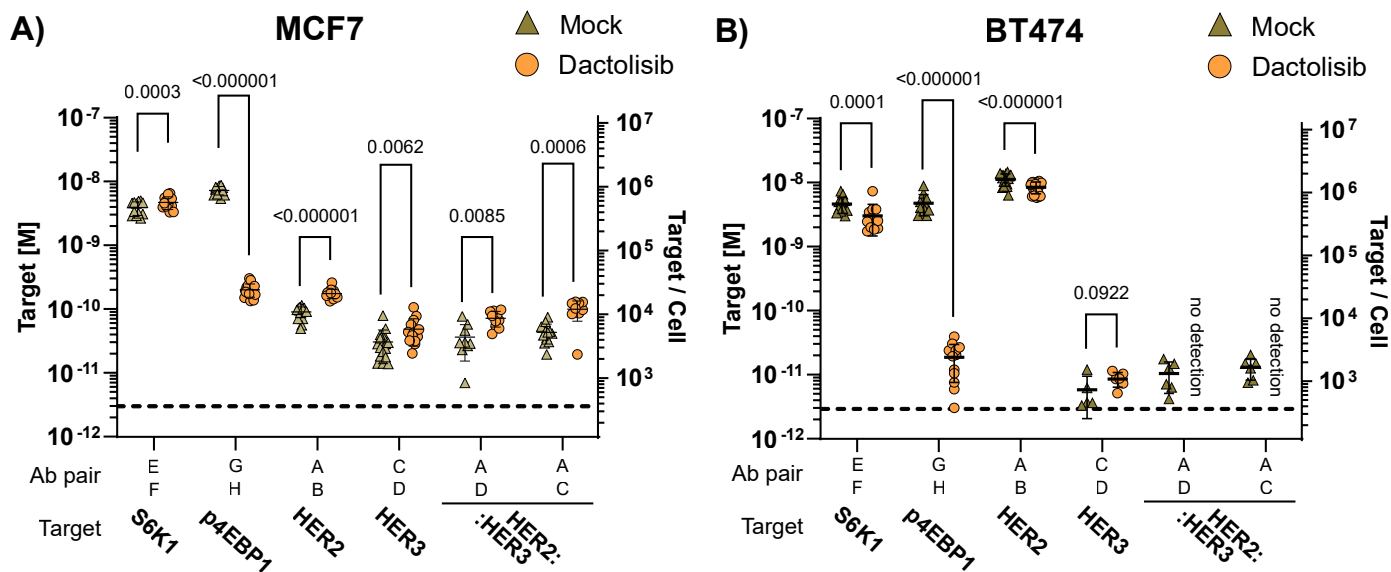
the western blot findings from Serra and colleagues, which demonstrated that PI3K inhibition leads to enhanced HER2/3 signaling and acquired ERK dependency in HER2-overexpressing breast cancer (4).



**Figure 4: Proof of concept of the splitPICO workflow.** The number of complexes for **A)** S6K1 and p4EBP1 and **B)** HER2, HER3, and HER2::HER3 were detected with QIAGEN's QIAcuity dPCR System in increasing amounts of MCF7 cells. The eight antibodies were applied concurrently, but detected in two different dPCR reactions: S6K1, p4EBP1 (Alpha), and HER2, HER3, HER2::HER3 interaction (Beta). The detected targets for each antibody pair are indicated in the X axis (see also Figure 1), dash denotes the non-sense antibody pairs rendering the expected readout to zero. The asterisk (\*) indicates a significant p-value of <0.05 above zero.

## Conclusion

Taken together the splitPICO workflow is a powerful and versatile tool for comprehensive signaling pathway analysis, providing unprecedented insight into cellular processes. By enabling the simultaneous detection and absolute quantification of multiple targets this method overcomes limitations commonly associated with traditional protein analysis techniques. It reduces the amount of input material required for detailed analysis while minimizing handling time and technical variability by simplifying sample preparation. In this application note, the robust capa-



**Figure 5: Absolute quantitative splitPICO results. A)** MCF7 and **B)** BT474 cells were treated with either mock (green triangles) or Dactolisib (orange dots). The left y-axis shows the molar concentration of the target protein in the binding reaction, while the right y-axis represents the target protein count per cell. The dashed line indicates the limit of detection. Note the self-confirmation reproducibility of the splitPICO detection in the case of AD and AC pairs, HER2::HER3 interaction. Also note that the low limit of detection (LOD) for protein per cell is 250.

ibilities of splitPICO were demonstrated through the precise and absolute quantification of HER2, HER3, and their interactions, along with the downstream effects on key proteins such as S6K1 and p4EBP1, especially in response to targeted treatments like Dactolisib. Although this demonstration utilized an 8-plex assay (detecting 8 antibodies simultaneously), the technology is highly scalable, making it adaptable for expanding the number of targets analyzed. This versatility positions splitPICO as an ideal solution for both basic research and diagnostic applications, offering significant potential to advance our understanding of complex cellular signaling pathways.

## Materials and Methods

### Antibodies

Monoclonal antibodies targeting non-overlapping epitopes of HER2, HER3, S6K1, p4EBP1 (T46/T37) were labeled with either PICOglue Alpha Labels (#PICO-000124 to 127 for S6K1 and p4EBP1 antibodies) or with PICOglue Beta Labels (#PICO-000128 to 131 for HER2 and HER3 antibodies) using the PICOglue Antibody Labeling Kit (#PICO-000111).

### Cell culture

The breast cancer cell lines BT474 and MCF7 were treated in the cell culture medium with either 5.6  $\mu$ M Dactolisib (#ab120882, Abcam) or DMSO (mock) for 4h. The indicated amounts of BT474 and MCF7 cells were lysed according to the PICO Amplification Core Kit User Manual. For the binding reaction, 2  $\mu$ l of cell lysate was mixed with a total of 2  $\mu$ l labeled antibodies at a concentration of 500 pM and incubated overnight. At least six technical replicates per sample were included.

### PICO Assay

For the dPCR step, we aimed for an average lambda of 0.15 and diluted the binding reaction accordingly, as recommended in the PICO Amplification Core Kit User Manual. Two master mixes, one with Alpha Primers and the other with Beta Primers were prepared. From the last dilution 1  $\mu$ l was transferred to each master reaction mix. 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 complexes were processed as described in the PICO Amplification Core Kit User Manual, incorporating both ABC compensation and label-

ing 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 complexes. Applying the appropriate statistical test, it was assumed that the data followed a normal distribution based on the theoretical statistical distribution of complexes (5).

## References

1. Sanchez-Vega, Francisco, Marco Mina, Joshua Armenia, Walid K. Chatila, Augustin Luna, Konnor C. La, Sofia Dimitriadou, et al. 2018. "Oncogenic Signaling Pathways in The Cancer Genome Atlas." *Cell* 173 (2): 321-337.e10. <https://doi.org/10.1016/j.cell.2018.03.035>.
2. Rimawi, Mothaffar F., Rachel Schiff, and C. Kent Osborne. 2015. "Targeting HER2 for the Treatment of Breast Cancer." *Annual Review of Medicine* 66:111–28. <https://doi.org/10.1146/annurev-med-042513-015127>.
3. Liu, Ta-Jen, Dimpy Koul, Tiffany LaFortune, Ningyi Tiao, Rui Jun Shen, Sauveur-Michel Maira, Carlos Garcia-Echeverria, and W.K. Alfred Yung. 2009. "NVP-BEZ235, a Novel Dual Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin Inhibitor, Elicits Multifaceted Antitumor Activities in Human Gliomas." *Molecular Cancer Therapeutics* 8 (8): 2204–10. <https://doi.org/10.1158/1535-7163.MCT-09-0160>.
4. Serra, V., M. Scaltriti, L. Prudkin, P. J. A. Eichhorn, Y. H. Ibrahim, S. Chandarlapaty, B. Markman, et al. 2011. "PI3K Inhibition Results in Enhanced HER Signaling and Acquired ERK Dependency in HER2-Overexpressing Breast Cancer." *Oncogene* 30 (22): 2547–57. <https://doi.org/10.1038/onc.2010.626>.
5. Gross, Tobias, Tobias Hundertmark, Villó Csiszár, András Attila Sulyok, Nina Gross, Maike Breiden, Niklas Kitschen, et al. 2024. "Lossless Single-Molecule Counting To Absolute Quantify Proteoforms." *bioRxiv*. <https://doi.org/10.1101/2024.03.19.585761>.

