# TARGETING HER2 PROTEIN IN INDIVIDUAL CELLS USING ICP-MS DETECTION AND ITS POTENTIAL AS PROGNOSTIC AND PREDICTIVE BREAST CANCER BIOMARKER

A. Fernández Asensio[a,b], M. Corte-Rodríguez[a], J. Bettmer[a], L.M. Sierra[b], M. Montes-Bayón[a]* and E. Blanco- González[a]*.

[a]Department of Physical and Analytical Chemistry. Faculty of Chemistry. University of Oviedo. C/ Julián Clavería 8, 33006 Oviedo, Spain. Instituto de Investigación Sanitaria del Principado de Asturias (ISPA).

[b]Department of Functional Biology (Genetic Area). Faculty of Medicine. University of Oviedo. C/ Julián Clavería 8, 33006 Oviedo, Spain. Instituto Universitario de Oncología del Principado de Asturias (IUOPA) and Instituto de Investigación Sanitaria del Principado de Asturias (ISPA).

Correspondence to: *montesmaria@uniovi.es or *eblancog@uniovi.es

## ABSTRACT

The human epidermal growth factor receptor 2 (HER2) is a transmembrane protein that has become one of the most specific prognostic and predictive biomarker of breast cancer. Its early detection is key for optimizing the patient clinical outcome. This work is focused on the detection of HER2 in individual cells using an antibody containing lutetium (Lu) as reporter group that is monitored by introducing the individual cells into the inductively coupled plasma mass spectrometer (ICP-MS). This Lu-containing antibody probe is used to label different breast cancer cell lines considered HER2 negative (MDA-MB-231) and positive (SKBR-3 and BT-474). Optimizations regarding the amount of the probe necessary to ensure complete labelling reactions are conducted in the different cell models. Concentrations in the range of 0.006 fg Lu/cell and 0.030 fg Lu/cell could be found in the HER2 negative and HER2 positive cells, respectively. In addition, the selectivity of the labelling reaction is tested by using two different metal-containing antibody probes for HER2 (containing Lu) and for transferrin receptor 1 (containing Nd), respectively, within the same cell population. Finally, the methodology is applied to the targeting of HER2 positive cells in complex cell mixtures containing

variable amounts of BT-474 and MDA-MB-231 cells. The obtained results showed the excellent capabilities of the proposed strategy to discriminate among cell populations. This finding could help for scoring HER2 positive tumors improving existing technologies.

**Keywords:** HER2, cell targeting, single cell ICP-MS, antibody labelling, breast cancer, biomarkers.

## 1. INTRODUCTION

Breast cancer is a heterogeneous disease with different intrinsic subtypes affecting women worldwide [1]. One of the subtypes is the so called HER2 positive, which correlates with the overexpression of the human epidermal growth factor receptor 2 (HER2) in the tumor cells, and accounts for 20-30% of the diagnosed breast cancers [2]. HER2 is a transmembrane protein with an intracellular tyrosine kinase domain and an extracellular domain that is normally expressed at low levels in the epithelial cells of many tissues. The aberrant expression of HER2, however, plays a crucial role in oncogenic transformation and tumorigenesis in different types of cancer [3]. For instance, breast cancers can have up to 25–50 copies of the *HER2* gene, and up to 40–100 fold increase in HER2 protein levels resulting in more than 2 million receptors expressed at the tumor cell surface [4]. Such overexpression has been shown to be correlated with the growth and progression of a very aggressive subtype of breast cancer (HER2 positive) [5,6]. Thus, the HER2 status (HER2 positivity) has become a biomarker of poor prognosis, associated with a high rate of recurrence and mortality in patients with breast cancer [7], and, most important, it is the sole marker for stratifying patients to anti-HER2–based therapies [7]. In addition, the diagnosis as HER2 positive breast cancer has also a predictive value as biomarker of response to a variety of therapy regimens including endocrine therapies, chemotherapy with different agents such as anthracyclines and taxanes, and HER2-targeted therapies [8–10]. Therefore, the early detection of HER2 positive breast tumor cells is crucial for optimizing clinical outcomes in the affected patients.

In this regard, two different types of methods are clinically used in the analysis of this prognostic and predictive breast cancer biomarker [11,12]: those based on the

detection of HER2 protein overexpression (protein levels), and those based on the detection of *HER2* gene amplification (gene copy numbers) or *HER2* gene expression (messenger RNA levels). Within the first group, the most commonly used technique in clinical practice is immunohistochemistry (IHC), which involves the detection of HER2 protein in formalin-fixed, paraffin-embedded (FFPE) sections of biopsy specimens using a primary antibody against the HER2 protein. The protein-antibody complex formed in cell membranes is subsequently visualized by adding a secondary antibody with a fluorescent or enzymatic label. Currently, four commercially available IHC testing kits have been approved by the American Food and Drug Administration (FDA) to evaluate HER2 status [13]. However, issues such as tissue fixation, choice of primary antibody, and determination of thresholds for reporting positive results, which is very much influenced by the observer, are critical aspects when using this strategy, as well as, the inability to generate quantitative data [14]. On the other hand, some efforts have been focused on the development of an enzyme linked immunosorbent assays (ELISA) to quantify the HER2 extracellular domain. This part of the protein can be excised by metalloproteases from the full-length HER2 and circulate freely in serum where the determination is commonly conducted [15]. However, there are still some uncertainties about the utility of HER2 extracellular domain levels in serum as a valid prognostic/predictive tool of HER2-positive breast cancers [16].

Regarding the detection of the *HER2* gene amplification, fluorescence in situ hybridization (FISH) is currently the most widely technique in the clinical setting to assess gene amplification and three different kits have been already approved by the FDA [13,17]. The technique, which is performed on FFPE tissues, uses fluorescent-labeled probes (oligonucleotides) to detect specific DNA fragments in cell nucleus by hybridization of complementary sequences. Although FISH is considered a more accurate and reliable technique than IHC, it is more expensive, time-consuming and technically demanding [12]. Therefore, alternative techniques based on the amplification of target DNA sequences by polymerase chain reaction (PCR) have been developed to assess *HER2* gene amplification, such as quantitative PCR (qPCR) and multiplex ligation dependent probe amplification (MLPA) [11,12]. Finally, the evaluation of HER2 status based on messenger RNA levels by reverse transcription-quantitative

polymerase chain reaction (RT-qPCR) [18], or by commercially available microarray-based test [19], has also been proposed. The implementation of all these techniques in clinical routine, however, still needs to be thoroughly examined.

In addition to the previously described methods, nowadays there is an increasing interest in the determination of HER2 status directly in individual cells, in particular in circulating tumor cells (CTCs) [20]. CTCs are defined as the small number of tumor cells spreading through the blood, after detaching from the primary tumor, and can be considered to be responsible for the establishment of distant metastasis [21]. After capture and enrichment from a blood sample, the detection of CTCs is based on the labelling of tumor cell-specific markers at the cell surface using specific antibodies containing fluorophores [21]. Thus, one of the advantages of detecting HER2 in CTCs is that the analysis could be done at the protein level directly on the cell surface. The use of CTCs as a surrogate marker to characterize a tumor eliminates the need for tissue collection, thus minimizing invasive procedures, since the analysis is performed in blood samples, which can be considered as liquid biopsies [22]. However, there is still a lack of clinical data to prove the validity of such measurements due to the instrumental challenges associated to single cell analysis, and to the extremely high sensitivity required to obtain conclusive data in patient samples. In this vein, recently, mass cytometry (MC) has emerged as the technology to allow detection and quantification of dozens of markers simultaneously in a single cell with enlarged sensitivity [23]. MC is therefore uniquely suited for multi-parameter analyses of heterogeneous biological samples, such as tumors. In this case, cells are incubated (“stained”) with antibodies containing metal isotopes as reporter groups (instead of fluorophores used in conventional flow cytometry) that bind targets of interest on and/or within the cell. Cells are then introduced individually into the inductively coupled plasma-time of flight mass spectrometer (ICP-TOFMS). The fast scanning capabilities of this type of mass analyzer facilitate the detection, almost simultaneously, of different isotopes (and elements) within a fast transient event corresponding to the ionization of a single cell in the plasma. Such features have been exploited for the imaging of HER2 protein levels in cell cultures and also in tissue samples, although not yet in the context of CTCs analysis [24,25].

This study makes use of an analytical strategy based on the combination of antibody labelled with lanthanide-containing polymeric chelates and single-cell ICP-MS detection, to study HER2 protein cellular targeting in suspensions of complex cell populations for future application in breast cancer prognosis and prediction. For this purpose, HER2 protein is targeted in individual cells using antibodies containing lanthanide probes, in combination with a triple quadrupole ICP-MS fitted with a single cell sample introduction system. The optimization of the recognition procedure is performed in different breast cancer cell models with different levels of HER2 protein (MDA-MB-231, SKBR-3 and BT-474). The selectivity of the labelling process towards HER2 and the sensitivity of the targeting assay to discriminate HER2 positive cells within a mixed cell population with different levels of the protein is critically studied.

## 2. MATERIALS AND METHODS

### *2.1 Instrumentation.*

All ICP-MS experiments during this study were performed using the triple quadrupole instrument iCAP TQ ICP-MS (Thermo Fisher Scientific, Bremen, Germany) using the oxygen-TQ mode for the measurement of phosphorous (mass shift from $^{31}P^{+}$ to $^{31}P^{16}O^{+}$ after reaction with oxygen in the reaction cell), and SQ-mode (single quadrupole-mode) for $^{175}Lu^{+}$ and $^{142}Nd^{+}$ monitoring. For the single cell experiments, the ICP-MS instrument was fitted with the Single Cell Sample Introduction System SC (SC-SIS, Glass Expansion, Weilburg, Germany). The cells were pumped using a microflow syringe pump SP101i (Florida, USA) fitted with a 1 mL Hamilton syringe (Nevada, USA) at 10 µL $min^{-1}$. The data were recorded in time-resolved analysis mode during 3 min per analysis using a dwell time of 5 ms. Under these conditions, only a single isotope could be measured in one run due to the sequential nature of the measurements in a quadrupole system. Instrumental conditions are summarized in Table S1.

The studies of the labelled antibody were carried out by connecting on-line the size exclusion chromatography (SEC), using an HPLC system Agilent 1260 equipped with a binary pump (Agilent Technologies, Tokyo, Japan), to the iCAP TQ ICP-MS instrument as elemental detector. The column for the separation was a Superdex 200 10/300 GL (300 mm x 10 mm i.d., GE Healthcare Bio-Sciences) that has a fractionation range from

10 to 600 kDa. An aliquot of 100 µL of a water-diluted labelled antibody solution was injected in the column. The chromatographic separation was performed in isocratic mode for 45 min using a mobile phase at a flow rate of 0.7 ml $min^{-1}$.

For cell counting, a Flow Cytometer Cytoflex S Beckman Coulter (California, USA) was used. The cell number was determined by absolute counting. For this aim, the peristaltic pump of the flow cytometer was calibrated at 60 µL $min^{-1}$. Forward and scattered light from the blue laser (488 nm) was registered in order to determine and count the intact cells according to their size and morphology, and to exclude cell debris.

### *2.2. Reagents and materials.*

All solutions were prepared using 18 MΩ·cm de-ionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Human HER2 antibody MAB9589 (Research Grade Trastuzumab Biosimilar CyTOF-ready) and the mouse anti-human for transferrin receptor 1 (TfR1) monoclonal antibody were purchased from R&D Systems (Minneapolis, MN, USA). The mobile phase for the SEC chromatography used for the characterization of the antibody consisted of a 50 mmol $L^{-1}$ ammonium acetate (Merck Millipore, Darmstadt, Germany) solution at pH 7.0, at a flow rate of 0.7 mL $min^{-1}$. The column was previously calibrated using protein standards (thyroglobulin 660 kDa, ferritin 450 kDa, immunoglobulin G 150 kDa, albumin 66 kDa and alfa-lactalbumin 15 kDa) obtained from Sigma-Aldrich (Madrid, Spain). Lutetium standard for calibration was purchased from Merck.

The antibodies were labelled using a Maxpar X8 Antibody Labelling Kit (Fluidigm, San Francisco, CA, USA), following the instructions of the manufacturer. For the reduction of the antibody, tris(2-carboxyethyl) phosphine (TCEP) was purchased from Sigma-Aldrich. For the purification steps, centrifugal filter units of 3 kDa and 50 kDa were used (Amicon Ultra 0.5 mL, Merck Millipore). For cell fixation, a buffered aqueous solution of formaldehyde 4% (v/v) (VWR Chemicals, Pennsylvania, USA) was used. Phosphate buffered saline (PBS), tris buffered saline (TBS) and bovine serum albumin were obtained from Sigma Aldrich.

### *2.3. Cell cultures.*

Human breast cancer cell line MDA-MB-231 (HER2 negative) was a kind gift from Dr. Santiago Cal (Department of Biochemistry and Molecular Biology, University of Oviedo, Spain). Human breast cancer cell line SKBR-3 (HER2 positive) was kindly provided by Dr. Xose Antón Suárez Puente (Department of Biochemistry and Molecular Biology, University of Oviedo, Spain), and human breast cancer cell line BT-474 (HER2 positive) was purchased from the American Type Culture Collection (ATCC, Manasas, USA). MDA-MB-231 and BT-474 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, LabClinics, Barcelona, Spain) supplemented with 10% (v/v) fetal bovine serum (Gibco, Life technologies, Madrid, Spain) for MDA-MB-231, and with 20% (v/v) fetal bovine serum and 0.01 mg $mL^{-1}$ bovine insulin (Invitrogen, Carlsbad, USA) for BT-474. SKBR-3 cells were grown in Roswell Park Memorial Institute 1640 Medium (RPMI 1640, Invitrogen, Fisher Scientific, Madrid, Spain) supplemented with 10% (v/v) fetal bovine serum. Culture medium was always additionally supplemented with 5 µg $mL^{-1}$ Plasmocin prophylactic (InvivoGen, Nucliber, Madrid, Spain). Cells were grown in T-25 flasks at 37°C in a 5% (v/v) $CO_2$ atmosphere. Then, the cells were washed with PBS (three times) and collected with Corning™ CellStripper Dissociation Reagent (Nueva York, USA).

### *2.4. Cell fixation and tagging.*

After collecting the cells with the CorningTM CellStripper Dissociation Reagent, the cell number was determined by flow cytometry and adjusted to $10^6$ cells per aliquot. The cell pellet was re-suspended in 500 µL buffered formaldehyde 4% (v/v) and incubated for 15 min at room temperature, to fix the cellular structure during the labelling procedure, followed by washing with 3% (w/v) BSA in PBS. After centrifugation for 5 min at 300 g the pellet was collect and re-suspended in 200 µL of the antibody solution in 3% (w/v) BSA in PBS. This suspension was incubated for 30 min at room temperature and washed 3 times with 500 µL PBS (up to a total volume of 1500 µL) and 5 more times with 500 µL TBS (up to a total volume of 2500 µL) to minimize the phosphorus background.

### *2.5. Cell mixtures.*

Cell mixtures were prepared after collecting the cells from the cultures and determining the cell number by flow cytometry. Mixtures were always done by adding adequate volumes of each cell line in order to obtain a final mixture of $10^6$ cells. After the cell mixture was done, cells were centrifuged and the cell mix pellet was either fixated and tagged, as previously described for single-cell analysis, or lysed for ELISA determination.

### *2.6. Analysis by commercial ELISA.*

HER2 was quantified by a commercial ELISA for comparison using the HER2 Human ELISA kit from Thermo Fisher Scientific. This is a sandwich immunoassay that uses a plate pre-coated with a capture antibody and a biotin-conjugated detection antibody. Streptavidin- horseradish peroxidase (STV-HRP) is added to the plate and bound to the detection antibody after the sandwich formation. Then, a substrate solution (3,3',5,5'-tetramethylbenzidine, TMB) is added, which reacts with the HRP to produce a blue coloration. Afterwards, a stop solution is added and the blue coloration turns into yellow. The intensity of this signal is measured spectrophotometrically at 450 nm and is proportional to the HER2 concentration.

For the ELISA, the cells were lysed using the cell extraction buffer included in the human Transferrin Receptor SimpleStep ELISA kit from Abcam (Cambridge, U.K.). After lysis and centrifugation the supernatant was discarded an the pellet was collected and solubilized in the buffer, incubated on ice for 20 min and centrifuged at 18,000 g for 20 min at 4ºC.

### *2.7. Data treatment.*

For data treatment of single cell suspension measurements, an established iterative procedure was followed, based on averaging the entire data set and collecting all data points that are three standard deviations (3σ) above the mean. The resulting data set after removing the selected events is rearranged and the procedure is repeated until no new data points are above the 3σ threshold. After the selection of the single cell signals, those higher than 3σ above their mean were discarded, as reported previously, in order to eliminate multiple-cell events [26].

## 3. RESULTS AND DISCUSSION

### *3.1. Labelling reaction in cell models.*

The procedure for the labelling of the antibody followed the one provided by the manufacturer, and was previously conducted in our laboratory for detection of transferrin receptor 1 (TfR1) [27]. In summary, the reduction is done under controlled conditions using TCEP in such a way that only the sulfhydryl groups of the hinge region of the antibody were reduced. Subsequently, the polymeric chelate and the metal ion probes (Lu) were introduced to react with the reduced antibody by maleimide-thiol reaction. The evaluation of the labelling reaction was conducted by coupling the SEC column to the ICP-MS to monitor the metal probe, using similar conditions to those published previously [27]. The chromatogram obtained monitoring the $^{175}Lu^{+}$ signal in the ICP-MS is shown in Figure 1. As can be observed, two different peaks are present in the chromatogram corresponding, by comparison to previously obtained data [27], to the labelled antibody (at about 16 min, corresponding to 65% of the total Lu peak area) and to the excess of the labelling reagents (at about 20 min). The Lu concentration in the injected solution was obtained by ICP-MS, and the antibody concentration was obtained spectrophotometrically, at 280 nm, by using a commercial immunoglobulin G (IgG) protein standard for calibration. Considering that the chromatographic peak corresponding to the Lu-labelled antibody corresponds to 65% of the total Lu signal, a stoichiometry of 22 Lu per mol of antibody were obtained, similar to previously obtained results using the same labelling kit [27].

The Lu-containing antibody was used to tag the HER2 protein in three different cell cultures (one HER2 negative and two HER2 positive). Single cell events monitoring $^{175}Lu^{+}$ and $^{31}P^{16}O^{+}$ were obtained for the three different cell models. As an illustrative example to compare results for HER2 positive and HER2 negative cells, Figure 2 shows the events detected for the cell line MDA-MB-231 (HER2 negative, panels A and C) and one of the two HER2 positive cell lines, the SKBR-3 (panels B and D). As can be seen, although the phosphorous signals (A and B) are comparable in both plots, the number of events and their heights in the case of Lu (C and D) are significantly different. The detection of some Lu events in the case of the MDA-MB-231 is due to the presence of HER2, at low levels, in most breast cancer cells, and even in normal non-tumor cells. The

intensity of these events is very close to the instrumental detection limit, calculated as three times the standard deviation of the continuous background (3σ) that was the criterion selected as the threshold for discrimination of cells events from the background noise.

The next step was the titration of the antibody by optimization of the amount of antibody added to every cell culture to ensure complete tagging of the HER2 molecules present on the cell surface, while keeping low background levels. For this purpose, different dilutions of the labelled antibody were used to tag the HER2, and the Lu per cell was quantified in each case. To select the starting antibody concentration, we considered the data obtained in previously published work [27]. From that point, we increase the concentration until no effect on the efficiency of the labelling reaction (fg Lu/cell) was observed. As usually done when conducting calibration curves, increasing antibody concentrations that double the previous one were used (2, 4 and 8 μg/mL) and 10 μg/mL was used to assure a sufficient excess.

For such quantification, the ICP-MS was calibrated using Lu inorganic standards. The instrumental response per concentration unit, was transformed into the mass of Lu per cell using the previously established equation (see SI). Different results were observed for the different cell cultures analyzed, as can be seen in Figure 3 where the three panels reveal the mass of Lu per cell observed in MDA-MB-231 (A), BT-474 (B) and SKBR-3 (C) cells, respectively. In the case of MDA-MB-231 cells, there are no significant differences in the mass of Lu per cell among antibody concentrations; since this cell line is considered as HER2 negative, the few receptors present on the cell surface can be tagged using the lowest antibody concentration. Higher antibody concentrations did not provide any changes in the Lu signal per cell, confirming the selectivity of the method towards HER2. In the case of BT-474 and SKBR-3 cells (Figure 3B and 3C, respectively), the mass of Lu per cell increases upon increasing the concentration of the antibody and, in both cases, stays constant applying an antibody concentration higher than 8 μg $mL^{-1}$. Thus, the antibody concentration of 8 μg $mL^{-1}$ seems to represent sufficient excess to tag all of the HER2 present in the cell surface of all the analyzed cell lines.

It is noteworthy from Figure 3 that the mass of Lu per cell is about 2-fold higher in the case of the SKBR-3 than in the BT-474, even when both cell lines are considered

equally positive with respect to HER2. These differences might be ascribed, however, to the cellular morphology exhibited by these two cell lines. The BT-474 cells might be slightly smaller in diameter than the SKBR-3 ones [28] and form colonies exhibiting robust cell to cell adhesion, while SKBR-3 cells are slightly larger [29] and can be distinguished by their grape-like appearance with poor cell to cell contact [30]. Therefore, the number of receptors per active area could be lower in the case of the BT-474 cells and, thus, the detected mass of Lu.

### *3.2. Analytical figures of merit*

To address method sensitivity, a number of Lu standards of different concentrations (from 0 to 2 ng $mL^{-1}$) were introduced into the system and the intensity obtained for each of them. Equation 1 (see SI) was used to obtain the sensitivity per dwell time to be able to calculate the mass of Lu entering the plasma per dwell time (considering that in every dwell time only one cell enters the system). Using this equation, it is possible to represent the Lu intensity for the standards versus the mass/dwell time of these standards. The slope of this graph is the sensitivity of the single cell method which turned to be 1800 cts/ng per dwell time. Regarding the limit of detection, it has been calculated as three times the standard deviation of the continuous background (3σ) that was the criterion selected as the threshold for discrimination of cells events from the background. The obtained detection limit turned out to be 0.002 fg Lu/cell.

The selectivity of the methodology towards HER2 was assessed by comparing the results on HER positive and negative cell lines (see Fig. 3). In addition, the selectivity of the labelling procedure using two antibodies tagged with different metals (Lu and Nd) within the same cell culture was also evaluated. For this aim, two different cell lines (MDA-MB-231 and SKBR-3) were labelled using two different antibodies, one of them against HER2 (labelled with Lu as metal probe) and another one against TfR1 (labelled with Nd as metal probe). Since the measurements were conducted with a TQ-ICP-MS, only sequential measurements could be conducted and, thus, monitoring of m/z 142 and 175 was carried out in separate runs of 180s. The obtained results can be observed in the box plot of Figure 4. As can be seen, the Lu intensity in the HER2 negative line (MDA-MD-231, orange box) is very low while in SKBR-3 (HER2 positive, blue box) is

significantly higher ($p<0.001$). This shows the selectivity of the method to discriminate HER2 positive and negative cell lines.

TfR1 is responsible for Fe uptake in cells through the Fe-loaded transferrin/TfR1 association and incorporation into cells. Since Fe is an essential element for cell survival, TfR1 is overexpressed in most tumor cells [27] and, therefore, it should be efficiently labelled in both cell lines (Nd-probe) although with different intensities due to the different expression level. Therefore, the obtained results confirm the selectivity of the labelling procedure for HER2 monitoring.

### *3.3. Targeting HER2 in cell mixtures.*

One of the main challenges of single cell experiments is to be able to discriminate among complex cell populations the presence of malignant phenotypes that might affect the future treatment of the disease ("to find a needle in a haystack"). This is currently done in routine clinical practice by IHC (as described before) on tissue specimens. However, the technique presents a few limitations, particularly in the assessment of HER2, regarding data normalization. In fact, several publications compared the data provided with this technique and those obtained by FISH for the same set of samples. Concordances ranging from 65% to 95% between both sets of data were obtained depending on the IHC scoring system [31]. Therefore, to prove the feasibility of the proposed methodology for HER2 targeting in mixed cell populations (e.g. extracted from a tissue specimen), different mixtures of HER2 negative (MDA-MB-231) and HER2 positive (BT-474) cells were prepared by mixing adequate volumes of the individual cultures. The mixtures contained approximately 100%, 80%, 50%, 20% and 0% HER2 positive (BT-474) cells with respect to the total cell number concentration ($1 \times 10^6$ cells/mL). After careful counting and mixing, they were further fixed and labelled with the Lu-probe. The mixtures were analyzed with the proposed strategy and the results can be seen in Figure 5, in a box plot corresponding to all the mixtures. The percentage of each of the different cell lines used for the measurements was selected to cover the whole range from 0% HER2 positive to 100% HER2 positive and including 5 data points. They should be sufficient to prove the suitability of the proposed strategy to detect HER2+ positive cells in mixtures of cells with different amounts of HER2, as can be found in tumoral samples.

As can be observed, the 0% BT-474 (100% MDA-MB-231) shows most events in the lower range of fg Lu/cell (< 0.01 fg Lu/cell), with the median about 0.006 fg Lu/cell and with a very small interquartile range (0.0028-0.0129 fg Lu/cell). As the percentage of BT-474 cells is increased in the mixture from 0% to 20, 50, 80 and 100% respectively, the median of the fg Lu/cell increases to 0.0157, 0.0188, 0.0271 and 0.0297, respectively (see Table 1). By plotting the median versus the %BT-474 (Fig. 6), a correlation coefficient of 0.955 can be obtained, showing the capability of the technique to discriminate among cell populations. Unfortunately, the main limitation of the proposed strategy derives from the fact that the BT-474 qualified as HER2 positive cell line generates Lu signals (and Lu concentrations) with larger dispersion (interquartile range of 100% BT-474 goes from 0.0217 to 0.0489 fg Lu/cell). This could be ascribed to the presence of phenotypic heterogeneity (higher and lower level of HER2 expression within the same cell population) that can only be detected by conducting single cell experiments [32].

### *3.4. ELISA measurements.*

In order to compare the developed strategy with existing quantitative assays, the different cell mixtures were analyzed using a commercial solid-phase sandwich ELISA assay. The results are summarized in Figure 7 and transformed into number of HER2 receptors per cell obtained by averaging the HER2 concentration found per well by the number of cells. As can be seen, there is a significant increase of the HER2 concentration in the cell lysate upon increasing the percentage of BT-474 in the mixtures (decreasing of MDA-MB-231), similar to what has been observed in Figure 5. The ELISA sensitivity, however, is slightly worse, as it can be observed in the mixtures containing 100% and 80% BT-474 that provide values not being statistically different ($p < 0.05$). In addition, the ELISA assay is applied to cell lysates, and this includes the part of the receptor that has been also endocyted and is present within the cell cytosol. Therefore, the concentration of HER2 obtained by this method does not only reflect the presence of this biomarker at the cellular surface, where it is biologically relevant.

## CONCLUSIONS

The present work shows the possibility of targeting HER2 protein in complex cell populations through the complementary use of Lu-labelled antibodies and SC-ICP-MS strategies. The optimization of the amount of the labelled antibody was clearly cell-type dependent, confirming the selectivity of the labelling reaction towards HER2. In addition, two different markers can be sequentially detected within the same cell population, corresponding to two receptors differently expressed on the cell surface using different metal labels. The discrimination among cell types within complex mixtures is possible using the proposed strategy, allowing the detection of a few HER2 positive cells within a majority of HER2 negative population. In comparison to the ELISA assay, the proposed SC-ICP-MS strategy provides the information about individual cells of the receptor present just at the cell surface, and that will be accessible to the treatment with anti-HER2 agents, which is the most used therapy for HER2 positive subtypes of tumors. The proposed methodology could serve to detect HER2 positive cells excised from biopsies in order to improve existing detection strategies.

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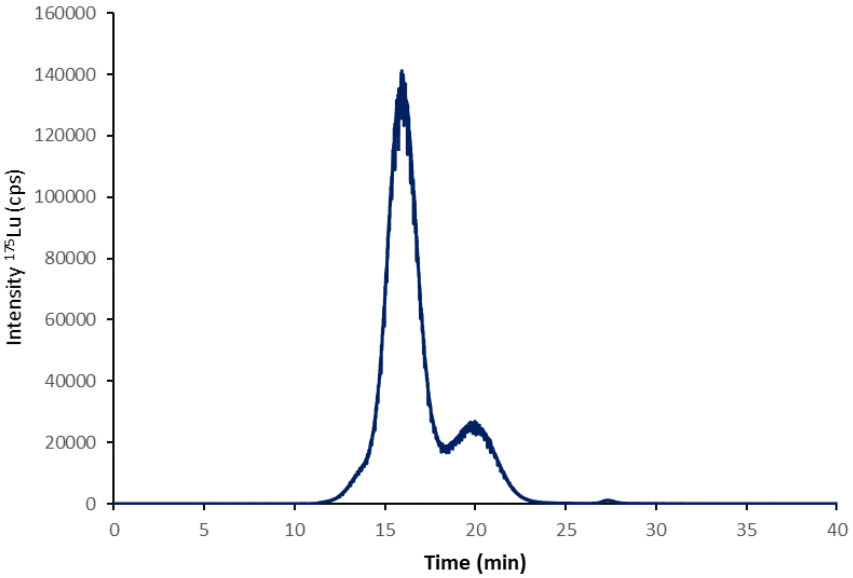


**Figure 1.** Chromatogram obtained by SEC-ICP-MS monitoring $^{175}$Lu of the anti-HER2 antibody after controlled reduction and labelling with Maxpar X8 Antibody Labelling Kit, using $^{175}$Lu as elemental probe.

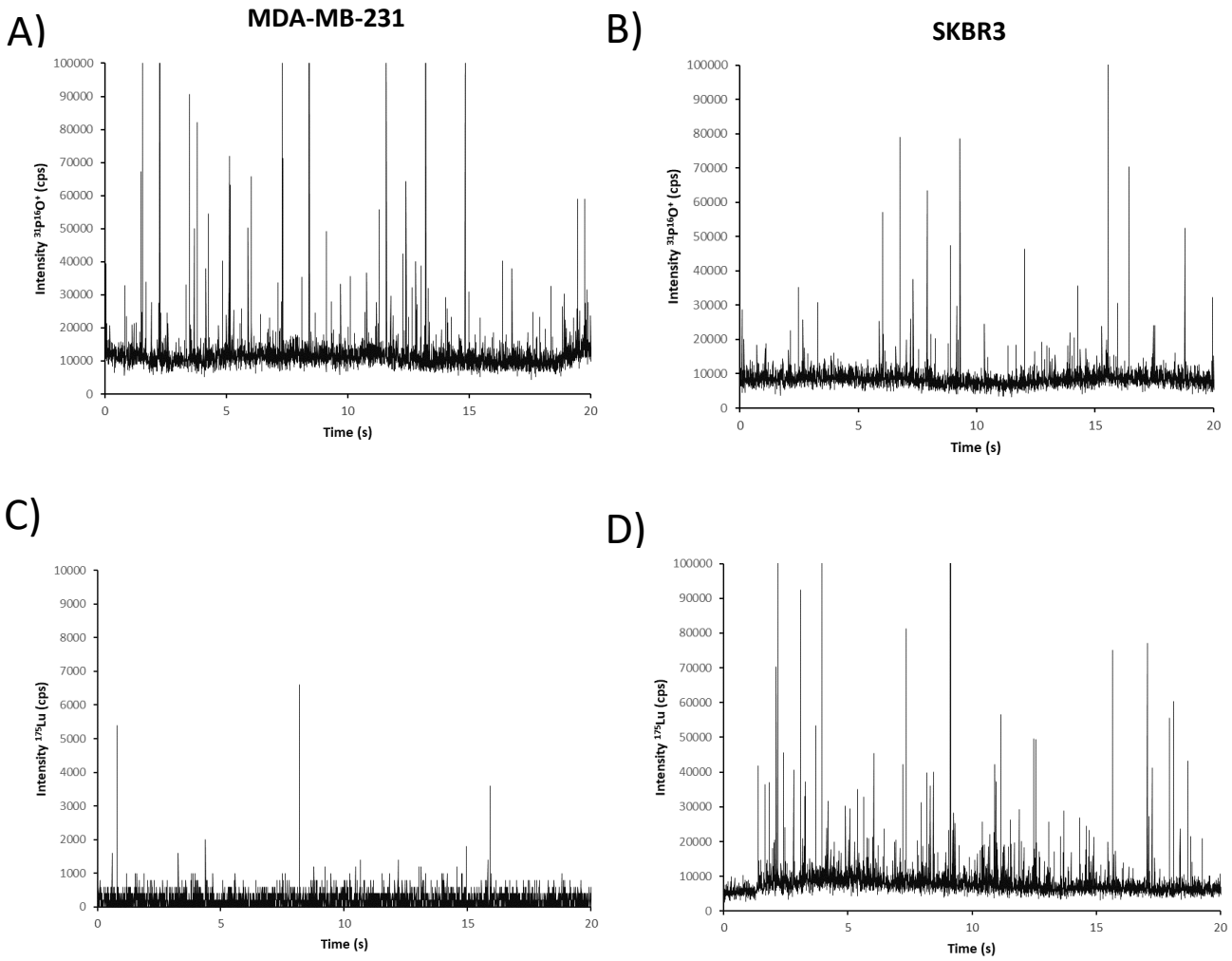


**Figure 2.** Single cell events registered for $^{31}P^{16}O^{+}$ and $^{175}Lu^{+}$, using TQ-ICP-MS, after labelling with the anti-HER2 antibody containing 175Lu as elemental probe. A) and C) MDA-MB-231 cell line; B) and D) SKBR-3 cell line.

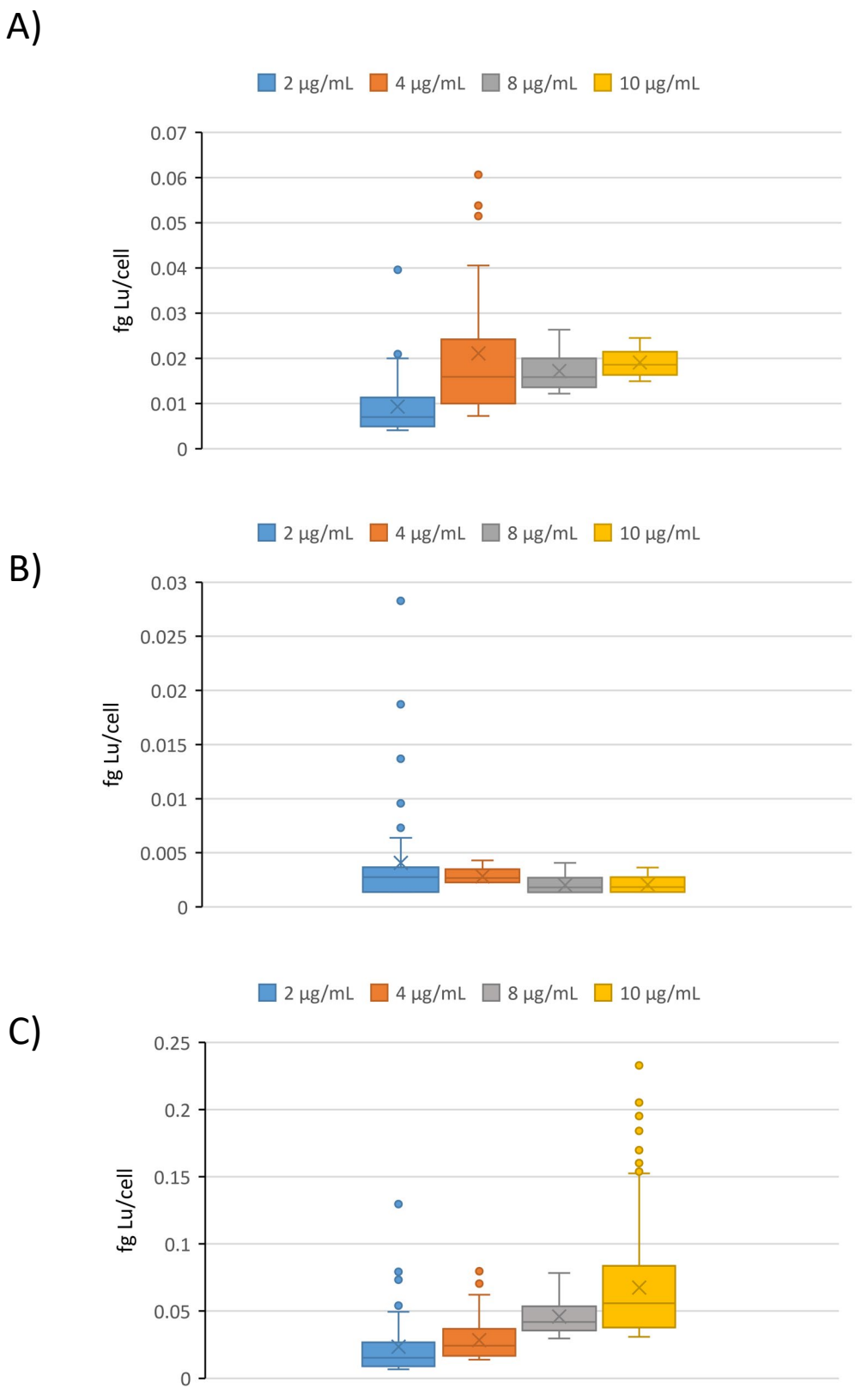


**Figure 3.** Box plot corresponding to the optimization of the antibody concentration used for the labelling of HER2 in the different cell lines by quantifying the Lu concentration per cell: A) MDA-MB-231, B) BT-477 and C) SKBR-3.

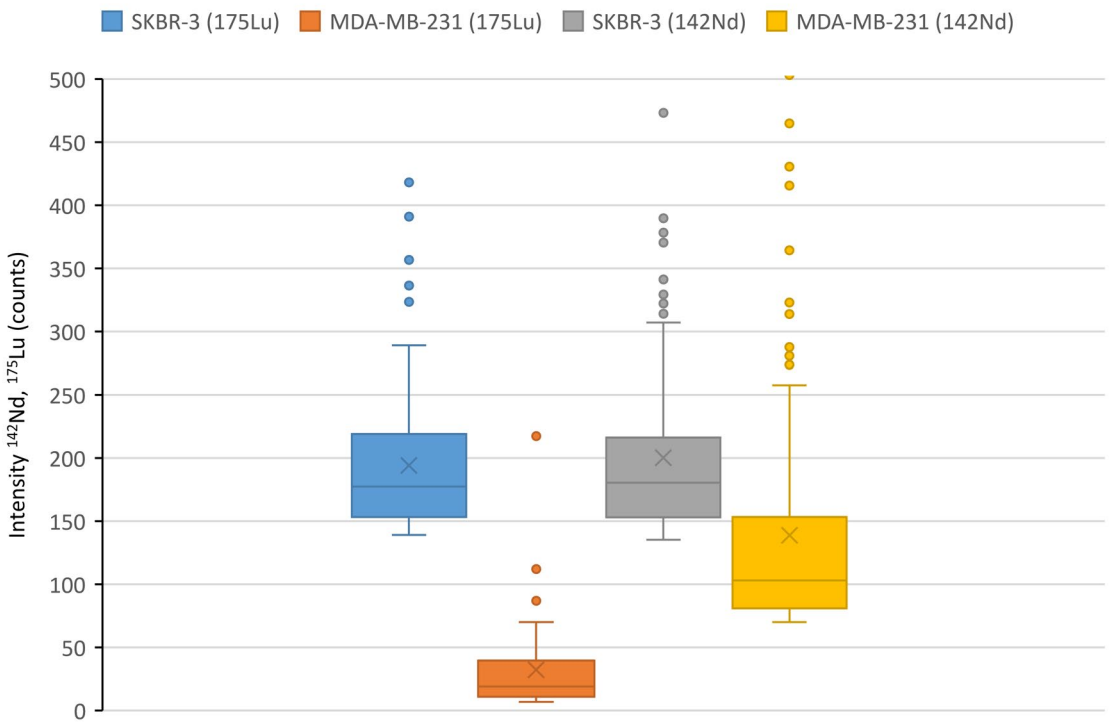


**Figure 4.** Box plot of the Lu and Nd signals corresponding to the simultaneous labelling of HER2 and TfR1, respectively, in cell lines MDA-MB-231 and SKBR-3.

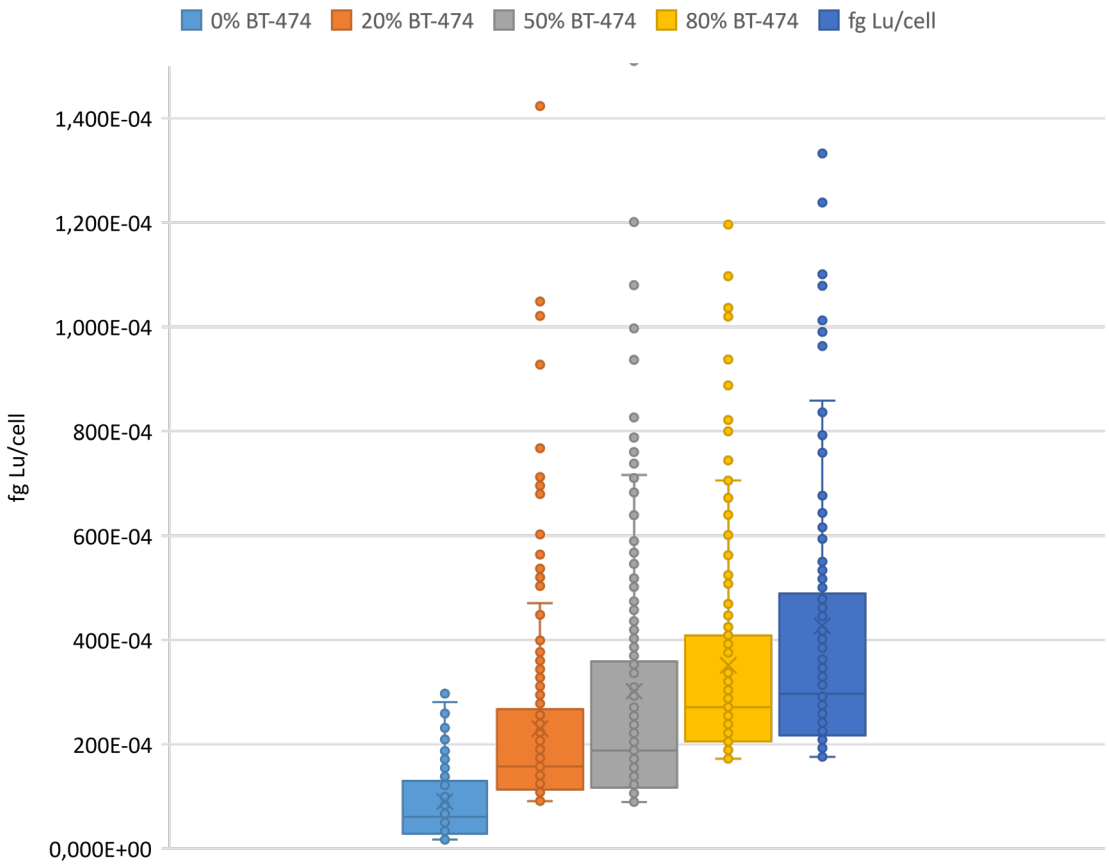


**Figure 5.** Box plot of the Lu concentration found in individual cells corresponding to cell mixtures containing 0, 20, 50, 80 and 100% BT-474 in MDA-MB-231.

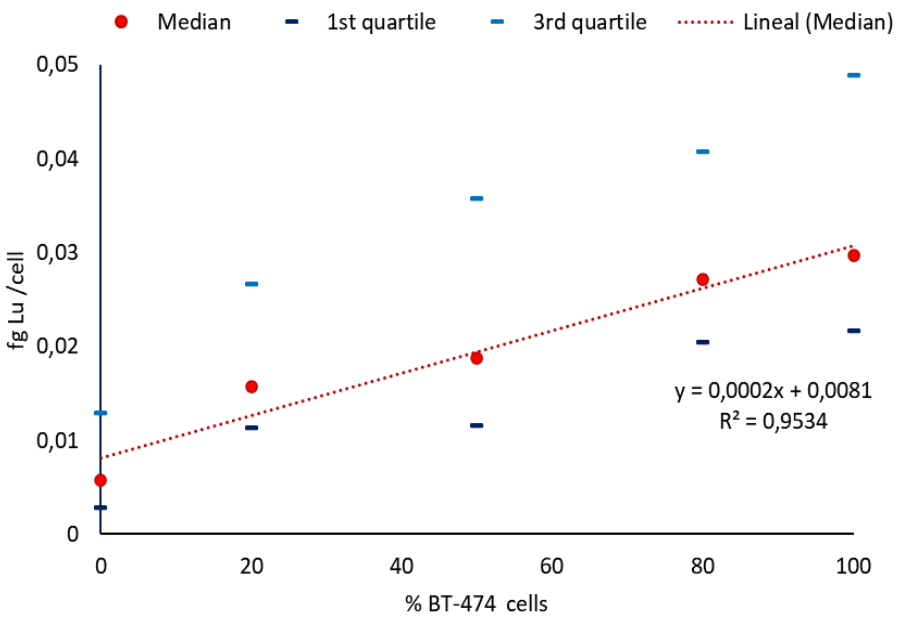


**Figure 6.** Calibration graph obtained by plotting the median of every box plot of Figure 5 and the corresponding 1st and 3rd quartiles versus the percentages of BT-474 cells in MDA-MB-231 cells.

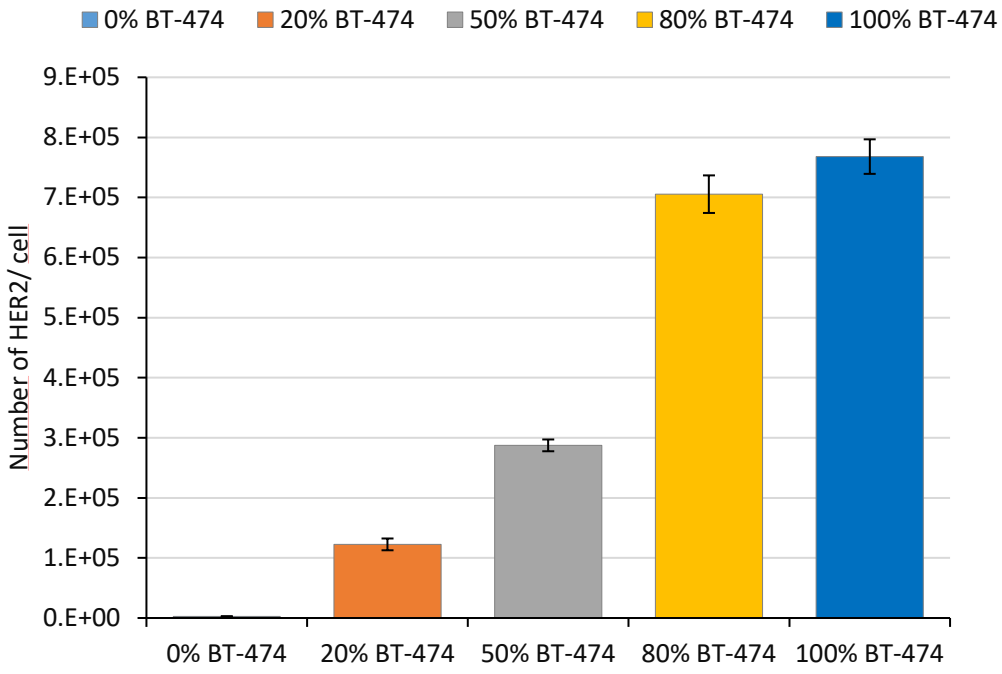


**Figure 7.** Number of HER2 receptors per cell found in cell lysates by commercial ELISA and corresponding to cell mixtures containing 0, 20, 50, 80 and 100% BT-474 cells in MDA-MB-231 cells.

**Table 1.** Extracted data from the box plot (Figure 5) obtained for the different mixtures of BT-474 and MDA-MB-231 cells.

| Sample | Median | Lower-upper quartile | Interquartile range |
|---|---|---|---|
| 100% BT-474 | 0.0297 | 0.0217-0.0489 | 0.0272 |
| 80% BT-474 | 0.0271 | 0.0205-0.0408 | 0.0203 |
| 50% BT-474 | 0.0188 | 0.0116-0.0358 | 0.0242 |
| 20% BT-474 | 0.0157 | 0.0113-0.0267 | 0.0154 |
| 0% BT-474 | 0.0058 | 0.0028-0.0129 | 0.0101 |