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Evaluation of the Dimerization Profiles of HER Tyrosine Kinases by Time-Resolved Förster Resonance Energy Transfer (TR-FRET)

Evelyne Lopez-Crapez, Alexandre Ho-Pun-Cheung, Patrick Garnero, and Hervé Bazin

Activation of receptor tyrosine kinases (RTK), such as those belonging to the human epidermal growth factor receptor (HER) family, occurs only after receptor dimerization, which is a crucial step for cellular signal transduction and diversification. The HER family includes four members (EGFR/HER1, HER2, HER3, and HER4) that can homodimerize or heterodimerize. Here, we describe immunoassays based on time-resolved Förster resonance energy transfer (TR-FRET) to profile EGFR-EGFR, HER2-HER2, and EGFR-HER2 dimers directly in tumor samples.

Key words Time-resolved Förster resonance energy transfer, Dimerization, Human epithelial growth factor receptor, Receptor tyrosine kinase, Antibodies

1. Introduction

HER expression is currently taken into account for tailored patients' management in oncology [1], but HER dimerization patterns in clinical samples may provide valuable information on receptor activation that cannot be inferred from their expression profiles. Assessment of HER dimerization is commonly performed in cultured cell lines by electrophoretic separation of cross-linked dimers or by co-immunoprecipitation [2]. However, these bio-chemical approaches are not suited for clinical studies because they require large amounts of material and cannot distinguish between HER protein direct and indirect interactions in which a third partner is also involved. Several new technologies have been developed to this aim. Particularly, the Duolink™ in situ proximity ligation assay [3], which is based on oligonucleotides attached to secondary antibodies and a complex combination of enzymatic reactions including ligation and rolling circle amplification, has been used to identify HER protein interactions [4]. This technology provides information on HER protein co-localization, but does not strictly indicate the existence of HER dimers. The VeraTag™ assay also depends on molecule proximity and the light-dependent release of a fluorescent tag quantified by capillary electrophoresis [5]. Unfortunately, this methodology is not commercially available and tests are carried out only by a centralized proprietary laboratory. Förster resonance energy transfer (FRET) is considered as the gold-standard fluorescence technique to detect molecular interactions. This approach relies on the transfer of energy between two fluorophores (a donor and an acceptor) with spectral compatibility. Excitation of the donor by an energy source triggers energy transfer to the acceptor only if they are in close proximity and the acceptor will then emit fluorescence. The use of long-lived fluorophores, such as those belonging to the lanthanide class, combined with time-resolved (TR) detection that introduces a delay between the excitation pulse and the emission detection allows the suppression of short-lived background fluorescence, thus improving the sensitivity of FRET-based assays [6]. Currently, a large variety of TR-FRET assays have been implemented for both clinical or research purposes using different biological fluids, such as serum and plasma, or cell cultures [7,8]. Recently we have developed antibody-based TR-FRET assays to characterize HER dimers directly in patients' tissue samples [9] (Fig. 1).

2. Materials

2.1. Fluorophores

1. *N*-Hydroxysuccinimide (NHS)-activated Lumi4® Tb cryptate (Cisbio Bioassays, Codolet, France).
2. NHS-activated d2 dye (d2-NHS) (Cisbio Bioassays).

2.2. Antibodies

1. Cetuximab (anti-EGFR monoclonal antibody): 5 mg/mL solution (Merck KGaA, Darmstadt, Germany).
2. Anti-EGFR monoclonal antibody (mAb) 528, purified from supernatants of the HB-8509 hybridoma cell line (American Type Culture Collection, Rockville, MD, USA).
3. Trastuzumab (anti-HER2 mAb): 20 mg/mL solution (Roche Pharma AG, Grenzach-Wyhlen, Germany).
4. Anti-HER2 FRP5 mAb (see Note 1).

2.3. Production of Antibody-Dye Conjugates

1. 100 mM phosphate buffer at pH 7 and pH 8 prepared with 1 M Na₂HPO₄ and 1 M NaH₂PO₄ stock solutions.
2. NAP-5™ columns (GE Healthcare Europe GmbH, Velizy-Villacoublay, France).

2.4. TR-FRET Assay Components

1. Phosphate-buffered saline (PBS; 10×): 1.35 M NaCl, 27 mM KCl, 80 mM Na₂HPO₄, 15 mM KH₂PO₄, pH 7.4.
2. Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Meylan, France).
3. Hoechst 33342, Trihydrochloride, Trihydrate: 10 mg/mL solution in water.
4. Incubation buffer: 10 % (100 mg/mL) bovine serum albumin (BSA) in 1×PBS with protease inhibitor cocktail. Dilute 10×PBS to 1×in ultrapure deionized water. In a 50 mL tube dispense 10 mL of 1× PBS and one tablet of complete, mini, EDTA-free protease inhibitor cocktail and vortex until completely dissolved (about 10 s). Add 1 g of powdered BSA and rock gently until BSA is completely dissolved. Filter consecutively through a 1 μm syringe filter and then a 0.2 μm filter unit. Prepare 2 mL aliquots and store at -20 °C.
5. Washing and reading buffer (WR buffer): 0.1% (1 mg/mL) BSA in 1× PBS with protease inhibitor cocktail. Prepare 10 mL of 1×PBS containing one tablet of complete, mini, EDTA-free protease inhibitor cocktail and then add 100 μL of incubation buffer (Subheading 2.4, item 5). Store at +4 °C.
6. PS microplates, 96-well, polystyrene, F-bottom, black.
7. 50 mL conical centrifuge tubes.

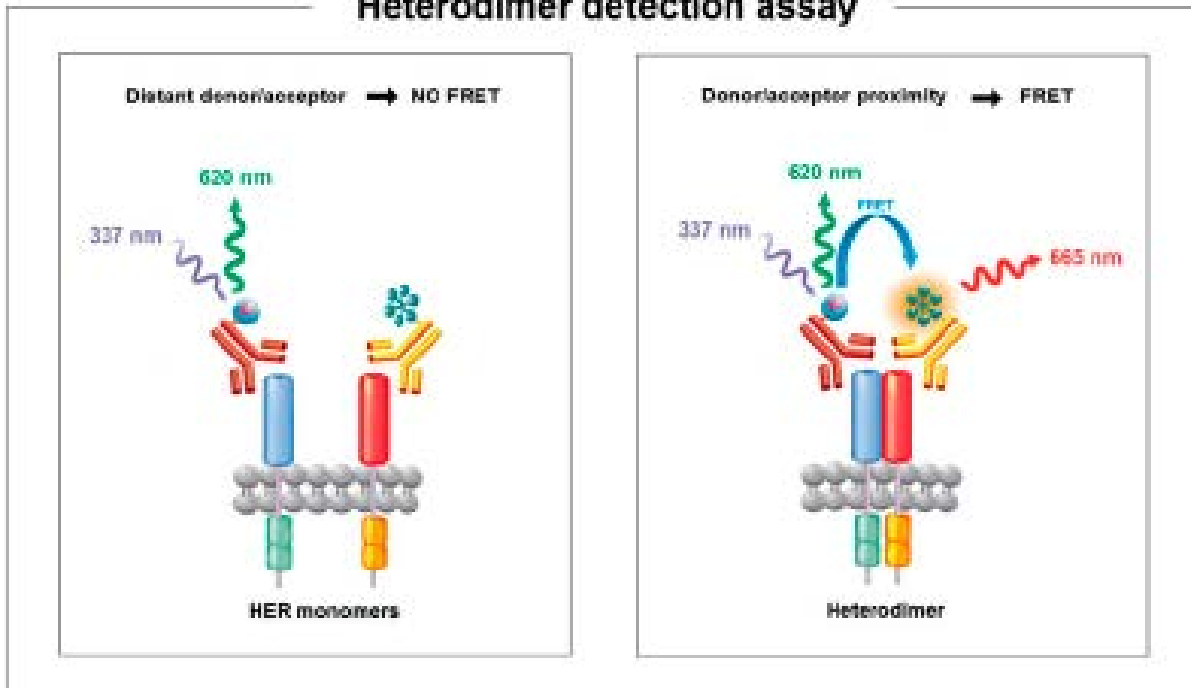
2.5. Xenograft Tumors

Nude athymic mice are xenografted with the ovarian carcinoma SKOV-3 (EGFR- and HER2-positive) cell line or the NIH/3T3 EGFR, NIH/3T3 HER2, and NIH/3T3 EGFR/HER2 cell lines, which are mouse embryonic fibroblasts stably transfected with EGFR and/or HER2, as previously described [8]. These tumors are used as positive and negative controls for the immunodetection of HER dimers.

2.6. Special Equipment

1. Sonicator.
2. Pherastar™ FS fluorimeter (BMG LABTECH, Champignysur-Marne, France) equipped with both an optic HTRF® 337/620/665 module for TR-FRET measurements and an optic FI 355/460 module for Hoechst 33342 fluorescence measurements.

Heterodimer detection assay



Homodimer detection assay

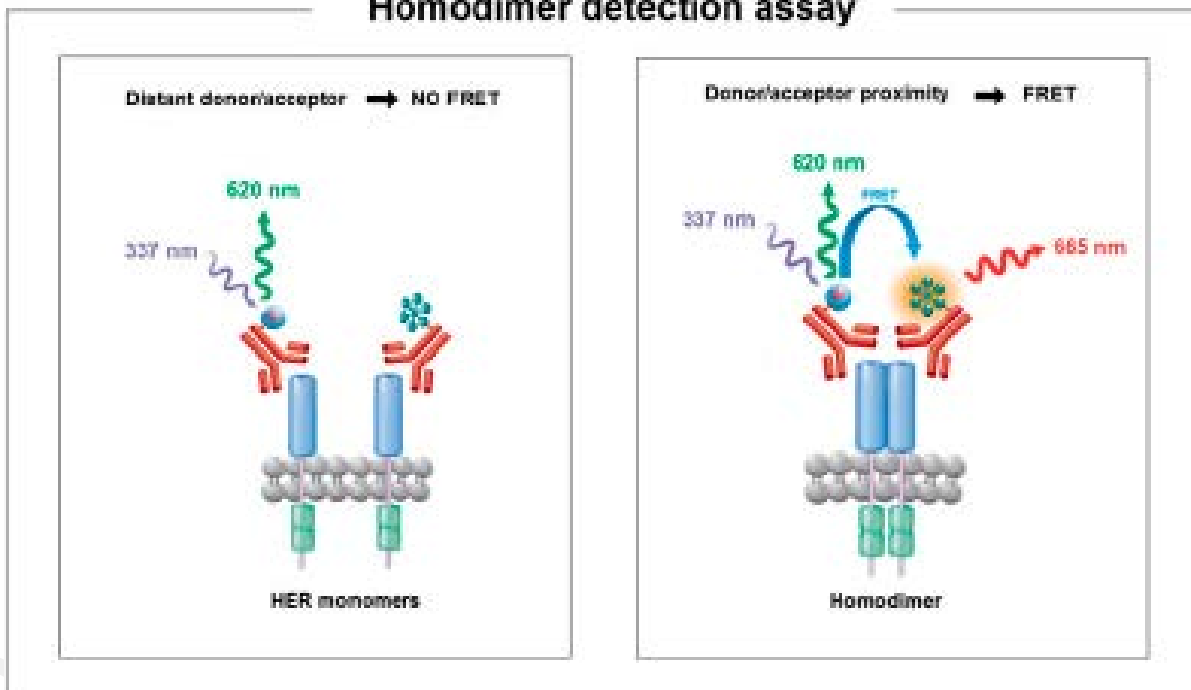


Fig. 1. Quantification of HER dimers using the TR-FRET technology. When an antibody conjugated with Lumi4[®] Tb (donor fluorophore) and an antibody coupled to d2 (acceptor fluorophore) bind to two receptors that form a dimer, the distance between donor and acceptor is small enough to allow FRET to occur. A portion of the energy captured by Lumi4[®] Tb during excitation at 337 nm is released through fluorescence emission at 620 nm, while the remaining energy is transferred to d2, which then can emit specific long-lived fluorescence at 665 nm. The fluorescence intensity measured at 665 nm in TR mode is proportional to the number of dimers, thereby allowing quantitative measurements.

3. Methods

3.1. Antibody-Lumi4® Tb Conjugates

1. Perform a buffer exchange of 100 μL of the cetuximab solution (see Note 2) using a NAP-5™ column. Prepare the column by removing the top and bottom caps to allow the excess storage buffer to flow through the column. Equilibrate the column with 100 mL of 100 mM phosphate buffer (pH 8). The phosphate buffer should enter completely in the gel bed by gravity flow. Add the cetuximab solution to the column and elute the antibody in the 300 μL fraction following the void volume; measure the antibody concentration by reading the absorbance at 280 nm (see Note 3).
2. Carry out the labeling step by adding 8 nmol of Lumi4® Tb-NHS cryptate to each nanomole of antibody solution and incubate at room temperature with roller agitation for 1 h.
3. Purify the labeled antibody by gel filtration on a NAP-5™ column equilibrated in 100 mM phosphate buffer (pH 7). Isolate the antibody-dye conjugate in the 400 μL fraction after the void volume.
4. Analyze the antibody-Lumi4® Tb conjugate fraction by determining the molar concentrations of Lumi4® Tb and antibody according to the Beer-Lambert law (see Note 4). The Lumi4® Tb concentration $[\text{Lumi4}^\circ\text{Tb}]$ is obtained from the following equation 1: $[\text{Lumi4}^\circ\text{Tb}] = A_{337} / \epsilon_{337} \text{ Lumi4}^\circ\text{Tb}$, where A_{337} is the measured absorbance at 337 nm and $\epsilon_{337} \text{ Lumi4}^\circ\text{Tb}$ is the molar extinction coefficient of Lumi4® Tb at 337 nm ($26,000 \text{ M}^{-1}\text{cm}^{-1}$). The antibody concentration $[\text{Ab}]$ is computed from equation 2: $[\text{Ab}] = [A_{280} - (A_{337}/7)] / \epsilon_{280}\text{Ab}$, where A_{280} is the absorbance measured at 280 nm, $(A_{337}/7)$ is the contribution of Lumi4® Tb at 280 nm and $\epsilon_{280}\text{Ab}$ the molar extinction coefficient of the antibody at 280 nm ($210,000 \text{ M}^{-1}\text{cm}^{-1}$).
5. Calculate the labeling ratio (LR1) according to equation 3: $\text{LR1} = [\text{Lumi4}^\circ\text{Tb}] / [\text{Ab}]$ (see Note 5).
6. Adjust the antibody-Lumi4® Tb conjugate concentration to 1.8 μM with 100 mM phosphate buffer (pH 7) and add BSA to reach a final concentration of 1 mg/mL. Distribute the antibody-Lumi4® Tb conjugate in 150 μL aliquots and store frozen (-20°C) until use.
7. Trastuzumab (see Note 2) and anti-HER2 FRP5 mAbs are labeled with Lumi4® Tb following the same protocol.

3.2. Antibody-d2 Conjugates

1. Perform buffer exchange of the cetuximab solution (150 μL) as described above (Subheading 3.1, step 1).
2. Carry out the labeling step by adding 5 nmol of d2-NHS for each nanomole of antibody solution and incubate at room temperature with roller agitation for 1 h.
3. Purify the labeled antibody on a NAP-5™ column equilibrated with 100 mM phosphate buffer (pH 7) as described above (Subheading 3.1, step 3). The antibody-d2 conjugate is eluted in the 300 μL fraction after the void volume.
4. Analyze the antibody-d2 conjugate fraction by determining the d2 and antibody molar concentrations according to the Beer-Lambert law (see Note 4). The d2 concentration $[\text{d2}]$ is obtained using the following equation 4: $[\text{d2}] = A_{650} / \epsilon_{650}\text{d2}$, where A_{650} is the measured absorbance at 650 nm and $\epsilon_{650}\text{d2}$ is the molar extinction coefficient of d2 at 650 nm ($225,000 \text{ M}^{-1}\text{cm}^{-1}$). The antibody concentration $[\text{Ab}]$ is computed from equation 5: $[\text{Ab}] = [A_{280} - (A_{650}/37.7)] / \epsilon_{280}\text{Ab}$, where A_{280} is the absorbance measured at 280 nm and $(A_{650}/37.7)$ the contribution of d2 at 280 nm.
5. Calculate the labeling ratio (LR2) according to equation 6: $\text{LR2} = [\text{d2}] / [\text{Ab}]$ (see Note 6).
6. Adjust the antibody-d2 conjugate concentration to 1.8 μM with 100 mM phosphate buffer (pH 7) and add BSA to reach a final concentration of 1 mg/mL. Distribute the antibody-d2 conjugate in 150 μL aliquots and store frozen (-20°C) until use.

7. Trastuzumab (see Note 2) and anti-EGFR 528 mAbs are labeled with d2 following the same protocol.

3.3. Clinical Tumor Cryosections

1. For each tumor sample stored at -80°C after surgical resection, cut consecutive 50 μm thick sections with a standard cryotome at -20°C (see Note 7). Place two sections directly in one 1.5 mL conical collection tube (see Note 8). Store prepared tubes at -80°C until use.
2. Each HER2 and EGFR dimer combination determination is carried out in duplicate by using two of the previously prepared tubes for each sample.

3.4. Xenograft Cryosections

1. Perform cryosections from xenograft tumors (used as negative and positive controls) as described above for clinical tumor samples.
2. For each dimer to analyze select adequate positive and negative controls (see Note 9).
3. Each dimer combination determination is carried out in duplicate by using two of the previously prepared tubes.

3.5. Immunodetection of HER Dimers

Do not allow tumor cryosections to thaw before step 3 of Subheading 3.5 . Carry out all procedures at room temperature unless otherwise specified.

1. Thaw aliquots of two antibody-dye conjugates that form a TR-FRET pair: cetuximab-d2/cetuximab-Lumi4[®] Tb (detection of EGFR-EGFR homodimers), trastuzumab-d2/trastuzumab- Lumi4[®] Tb (HER2-HER2 homodimers) or 528- d2/FRP5-Lumi4[®] Tb (EGFR-HER2 heterodimers).
2. For each dimer determination, prepare a TR-FRET master antibody mixture containing equal volumes of the two antibody-dye conjugates that form the desired TR-FRET pair (the concentration of each antibody-dye conjugate solution should have been adjusted to 1.8 μM ; see Subheading 3.2 , step 6).
3. Add 170 μL of incubation buffer to each tube with cryosections and then 10 μL of the prepared TR-FRET antibody mix (see Note 10).
4. Cap tubes securely and mix by vortexing for 5 s (see Note 11).
5. Prepare the standard solution by pipetting 20 μL of TR-FRET antibody mix into a 0.5 mL polypropylene tube and adding 340 μL of WR buffer. Mix by vortexing.
6. Protect tubes (samples and standard) from light and incubate overnight at 37°C in a shaking incubator.
7. The next morning, thaw the concentrated Hoechst 33342 solution for 20 min with protection from light (see Note 12).
8. Prepare 1:2 serial dilutions for the standard curve as follows: pipette 250 μL of WR buffer into eleven 0.5 ml tubes. Then pipette 250 μL of standard solution (see Subheading 3.5, step 5) into the first tube and mix. Pipette 250 μL of this mixture into the second tube and mix. Repeat the procedure (using 250 μL) for the other nine tubes to complete the standard curve points. Prepare a 12th tube containing only 250 μL WR buffer.
9. Dilute the concentrated Hoechst 33342 solution to 0.1 mg/mL with WR buffer.
10. Add 20 μL of diluted Hoechst 33342 solution to each control and clinical sample and mix by vortexing for 5 s (see Note 11).
11. Protect tubes from light and incubate controls and clinical samples at room temperature for 15 min.
12. Centrifuge tubes at $12,000 \times g$ for 3 min and discard supernatants (see Note 13). Resuspend pellets in 200 μL of WR buffer.
13. Repeat step 12 two more times. The third time, resuspend pellets in 250 μL of WR buffer.

- Sonicate controls and clinical samples at 80 W for 5 s (see Note 14). Mix by vortexing.
- Load 100 μ L of standards, WR buffer, positive and negative control, and clinical samples into two consecutive wells of a microtiter plate for duplicate measurements (Fig. 2) (see Note 15).

3.6. TR-FRET and Hoechst 33342 Signal Measurements

- Read the TR-FRET and Hoechst 33342 fluorescence signals straightaway (see Note 16).
- Measure the TR-FRET signal of samples and standards by reading the fluorescence at 620 and 665 nm in TR mode (delay, 60 μ s; time gate, 400 μ s) upon 337 nm excitation.
- Measure the Hoechst 33342 fluorescence intensity of control and clinical samples by reading the fluorescence at 460 nm in well scanning mode upon 355 nm excitation.

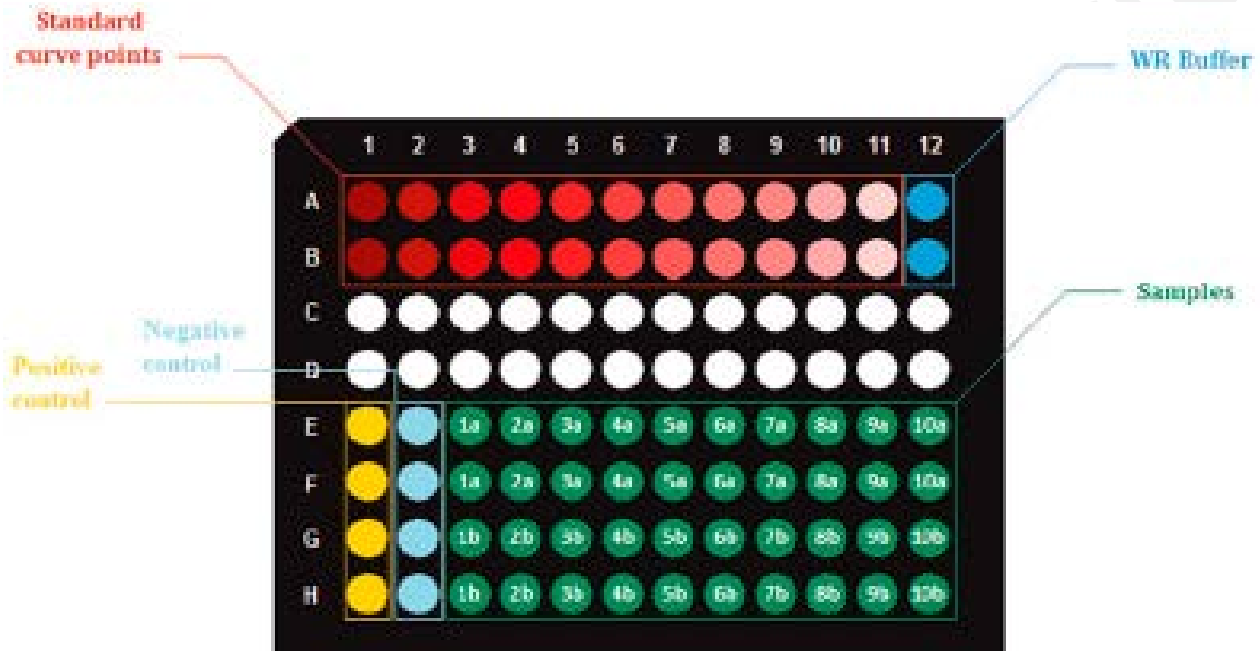


Fig. 2 Example of a 96-well plate layout for the analysis of one dimer type in ten clinical tumor samples. Biological duplicates are indicated as a and b.

3.7. Data Normalization

- Correct the measured fluorescence emissions (F) at 665, 620 and 460 nm for background fluorescence using the formula $F_{\text{corrected}} = F_{\text{sample}} - F_{\text{background}}$. The F background corresponds to the mean fluorescence value of the WR buffer at the corresponding wavelength. These corrected values ($F_{665\text{nm}}$, $F_{620\text{nm}}$ and $F_{460\text{nm}}$) are used for data normalization.
- Construct a standard curve by plotting the 665 nm fluorescence emissions of the standard curve points ($F_{665\text{nm}}$) on the y-axis against their 620 nm emissions ($F_{620\text{nm}}$) on the x-axis. Generate the straight-line equation for that line (Fig. 3).
- Use the equation of the standard curve to determine for each sample the fluorescence contribution from Lumi4[®] Tb at 665 nm ($F_{665\text{Tb}}$) using their $F_{620\text{nm}}$ as the X value in the equation.
- Calculate the sample TR-FRET signals ($\Delta F_{665\text{nm}}$) using the formula $\Delta F_{665\text{nm}} = F_{665\text{nm}} - F_{665\text{Tb}}$.
- Normalize the TR-FRET signal to the DNA content of the tissue sample using the formula $\text{normalized } \Delta F_{665\text{nm}} = \Delta F_{665\text{nm}} / (F_{460\text{nm}} / 100,000)$. The normalized TR-FRET signal is expressed in fluorescence units (see Table 1 for an example of HER2- HER2 analysis in breast tumor samples).

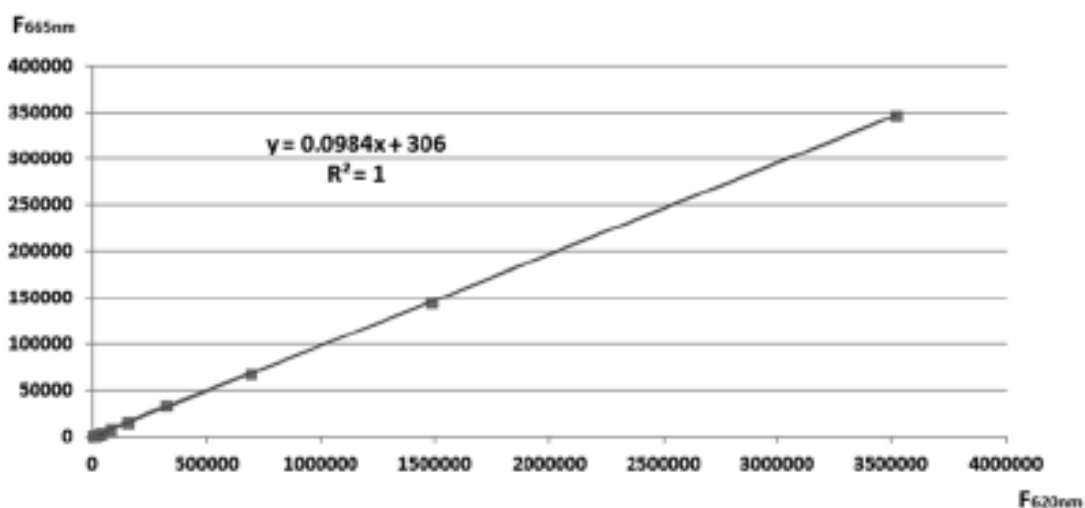


Fig. 3 Typical standard curve for the assessment of the Lumi4® Tb fluorescence contribution at 665 nm

Table 1. TR-FRET detection of HER2-HER2 homodimers in ten breast tumors

	NIH/3T3											
	SKOV-3	EGFR	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Normalized	107,890	0	12,958	511	0	0	0	35,369	0	14,992	655	0
ΔF_{665nm}	95,848	0	12,730	447	0	0	0	37,370	0	14,580	405	0
(FU)	94,457	0	10,970	697	0	0	0	30,944	0	13,170	880	0
	81,856	0	10,885	554	0	0	0	30,863	0	12,756	559	0

4. Notes

1. The anti-HER2 FRP5 mAb was kindly provided by Nancy Hynes (Basel, Switzerland) (ref.: Harwerth, I. M., Wels, W., Marte, B. M., and Hynes, N. E. (1992) *J Biol Chem* 267, 15160–7)
2. Commercial cetuximab is supplied in glycine-based buffer and trastuzumab contains L-histidine: both molecules must be removed before labeling because they react with NHS-activated dyes.
3. Buffer exchange using NAP-5™ columns is conservative and antibody recovery reaches 95 %.
4. For calculating the fluorophore and antibody concentrations we assume that the molar extinction coefficient of each moiety is unchanged in the antibody-dye conjugate.
5. Antibody-Lumi4® Tb conjugates contain an average of six molecules of Lumi4® Tb per antibody molecule.
6. Antibody-d2 conjugates contain an average of 3.6 molecules of d2 per antibody molecule.
7. Check the tumor cell content of each clinical sample under study by histologic examination of 5 µm-thick cryosections stained by hematoxylin and eosin. Two control slides should be prepared: one before the first cryosection and one after the last cryosection performed for dimer analysis. For specimens displaying less than 75 % of tumor tissue, tumor enrichment may be obtained by macrodissection to separate tumor tissue from the surrounding stroma and healthy tissue.
8. Prevent tissue thawing by cooling in advance all material (for instance, collection tubes) at -20 °C.
9. For EGFR-EGFR profiling, xenograft tumors from NIH/3T3 EGFR and NIH/3T3 HER2 cells are used as positive and negative controls, respectively. For HER2-HER2 homodimer detection, xenograft tumors from SKOV-3 and NIH/3T3 EGFR cells are used as positive and negative controls, respectively. For EGFR-HER2 heterodimer analysis, xenograft tumors from NIH/3T3 EGFR/HER2 and NIH/3T3 HER2 cells are used as positive and negative controls, respectively.

10. Perform this step as quickly as possible and do not analyze more than ten clinical samples at the same time.
11. At this step make sure that cryosections are submerged in solution and spin tubes briefly if necessary.
12. Upon reception, the concentrated Hoechst 33342 (10 mg/mL) solution should be aliquoted in polypropylene tubes and stored at -20°C .
13. To prevent pellet loss, use a P200 pipette and remove carefully the supernatant.
14. Before use, wash the sonotrode by immersing the extremity in a 20 mL tube containing refrigerated water. After each sample sonication, rinse carefully the sonotrode in the 20 mL tube with refrigerated water and dry with Kimwipes® disposable wipers.
15. For each dimer combination (HER2-HER2, EGFR- EGFR, and EGFR-HER2) prepare a new 96-well plate with the same layout (standard curve points, WR buffer, positive and negative controls, and clinical samples in duplicate).
16. TR-FRET and Hoechst 33342 fluorescence should be measured as quickly as possible following the distribution of samples in the 96-well plate.

Acknowledgments

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