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Methods for radiolabelling of monoclonal antibodies.

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Running head: Radiolabelling of antibodies

Summary

The use of radionuclide labels allow to study the pharmacokinetics of monoclonal antibodies, to control the specificity of their targeting and to monitor the response to an antibody treatment with high accuracy. Selection of label depends on the processing of an antibody after binding to an antigen, and on the character of information to be derived from the study (distribution of antibody in the extracellular space, target occupancy or determination of sites of metabolism). This chapter provides protocols for labelling of antibodies with iodine-125 (suitable also for other radioisotopes of iodine) and with indium-111. Since radiolabelling might damage and reduce the immunoreactive fraction and/or affinity of an antibody, the methods for assessment of these characteristics of an antibody are provided for control.

Key words: direct radioiodination, indirect radioiodination, CHX-A''DTPA, indium-111, iodine-125, immunoreactive fraction, saturation assay, LigandTracer.

1. Introduction

Following fate of antibodies in vivo can provide important information during preclinical development of antibody- based therapies, during clinical trials and, with the advent of personalized medicine, in daily clinical practice. The use of radioactive labels for antibodies facilitates such studies, because it is easy to quantify radioactivity concentration. Moreover, current imaging techniques, such a single photon emission computed tomography (SPECT) and positron emission tomography (PET), permit to visualise and quantify distribution of radioactivity in vivo by non-invasive procedures. Radiolabelled antibodies can be used during preclinical development for investigation of pharmacokinetics and targeting properties, dose finding and evaluation of response of treatment, hence speeding-up the process of drug discovery. The dose finding can be facilitated in clinical trials, reducing the number of patients treated with suboptimal doses of antibodies. In addition, the radiolabeled antibodies can be used for selection of patients who have tumours expressing a particular antigen and may benefit from particular therapy (*1*).

It has to be taken into account that labelling chemistry can influence cellular retention of radioactivity. Majority of antibodies are internalized after binding to cell-surface antigens, either by clathrin-dependent endocytosis or due to the normal turnover of cellular membrane constituents via non-clathrin-dependent endocytosis (*2, 3*). After internalization and translocation into lysosomal compartment, antibodies are proteolytically degraded. In vitro studies have demonstrated that the fate of a radioactive label after proteolytic degradation depends on lipophilicity of radiocatabolites (*4, 5*). Lipophilic radiocatabolites can diffuse through phospholipide lysosomal and cellular membranes, and leave the malignant cells. Such fate is typical for vast majority of radiohalogen labels (*6*). If the radiocatabolites are bulky hydrophilic (e.g. charged) molecular moiety, they would be retained inside the cells before

excretion by relatively slow externalization. The radiolabels, which are trapped inside the cells after internalization and degradation of targeting protein, are called “residualizing” or “trapped”. Radiometals are mainly residualizing labels (7) since their radiocatabolites are polar and often charged. Accordingly, different labels show different aspects of antibody distribution in vivo. Radioactivity associated with non-residualizing halogen labels reflects distribution of an antibody in blood, in extracellular space and bound to membranes of target cells or to extracellular matrix. Radioactivity associated with residualizing radiometal labels reflects, along with distribution of antibody outside cells, the amount of antibody, which has been internalized and degraded inside the tumour cells and by catabolizing organs.

Combination of both residualizing and non-residualizing labels for the same antibody provides the most complete information concerning its fate in vivo. Resolving gamma-spectra of radiometal and radiohalogen labels, one can derive such information from a single biodistribution study, upon co-injection in the same animal of an antibody labelled in two different ways (8).

One has to be aware that radiolabelling can influence a binding capacity of an antibody. For example, an oxidative radioiodination of antibodies using Chloramine-T (9) is the most commonly used method due to its robustness and simplicity. Radioiodide is oxidized in situ with subsequent attack of nucleophilic side-chains of a protein. A predominant site of electrophilic iodination at physiological pH is tyrosine (10). It was found (11, 12) that tyrosine residues are over-represented in complementarity determining regions (CDR) of antibodies. Iodination of tyrosines in CDR might decrease antigen binding capacity of antibodies (12). Lysines are presented in CDR to much lesser extent (13), and indirect halogenation, which is based on coupling of radiolabelled precursor to ω -amino groups of lysines, is often safer for proteins. However, over-modification of lysines might be also

unfavourable for binding capacity and biodistribution properties of radiolabelled antibodies. For this reason, one should take care that a number of pendant group (for radioiodination) or chelators (for radiometal labelling) should not exceed four-five per a protein molecules. After labelling of an antibody, the functional properties of antibody binding should be quantified to verify that adequate binding and acceptable immunoreactive fraction (IRF) is retained. The minimum level of quality control is to verify that the labelled protein interacts with the intended target. Since the binding properties of the unlabelled protein may be unknown, the value produced by the binding assay may serve as a characteristic of the labelled product, which can be followed over time to prove consistent performance of the labelling protocol. The saturation assay, which estimates the equilibrium dissociation constant K_D is a representative binding assay that performs well for interactions that reach equilibrium within one to a few hours. Real-time interaction analysis is a novel and more precise method for quantifying details of the binding characteristics. It is advantageous for high-affinity interactions, and requires less work than the manual binding assays, but relies on access to specialized equipment.

Another aspect of labelling quality is the immunoreactive fraction, i.e. the fraction of the labelled product, which is capable of binding to the target. The Lindmo assay (*14*) is the most commonly used method for assessing immunoreactive fraction.

In this chapter, we provide a description of two methods of radioiodination (a direct radioiodination using Chloramine-T (Fig.1 A) and indirect radioiodination using N-succinimidyl 4-thrimethylstannylbenzoate (Fig. 1B)) and labelling with ^{111}In using CHX-A''-DTPA (Fig. 1C). The most commonly used method for determination of affinity (saturation assay) and immunoreactive fraction (Lindmo analysis) of radiolabelled antibodies are also provided. In addition, a new method for determination of affinity of targeting proteins to living cells, LigandTracer analysis, is described. Besides an affinity value, this method

provides information concerning association and dissociation kinetics of an antibody interaction.

[Fig 1 near here]

^{125}I ($T_{1/2} = 60$ d) is the most commonly used radionuclide for preclinical studies (in vitro experiments, biodistribution in small rodents using direct ex vivo measurements, imaging in mice). This nuclide combines a long half-life with low radiation dose to personnel. The same protocol may be used for radioiodination using ^{131}I ($T_{1/2} = 8$ d) (for e.g. radionuclide therapy, or for dual-label biodistribution studies), ^{123}I ($T_{1/2} = 13.3$ h) (SPECT imaging) and ^{124}I ($T_{1/2} = 4.18$ d) (PET imaging). ^{111}In ($T_{1/2} = 2.8$ d) is a commercially available radiometal. Its half-life is compatible with biokinetics of intact IgG. ^{111}In is suitable for in vitro experiments, biodistribution in small rodents using direct ex vivo measurements, imaging in mice, as well as for clinical imaging using SPECT/CT.

2. Materials

2.1. Purification of an antibody

1. Milli-Q/ELGA water
2. NAP-5 column (GE Healthcare)
3. Eppendorf tubes (1.7 ml)
4. Antibody solution
5. Automatic pipette 1 ml, pipette tips.
6. Lyophilizing machine (freeze dryer)

2.2. Direct radioiodination of an antibody with ^{125}I using Chloramine-T

1. Milli-Q/ELGA water
2. Electronic balance (0.1 mg)

3. Freeze-dried antibody
4. NAP-5 column (GE Healthcare)
5. Eppendorf tubes (1.7 ml)
6. ^{125}I stock solution (GE Healthcare or PerkinElmer)
7. Chloramine-T trihydrate, sodium chloro-(4-methylphenyl)sulfonylazanide (Sigma or Merck)
8. Sodium metabisulfite, $\text{Na}_2\text{S}_2\text{O}_5$ (Sigma or Merck)
9. Automatic pipette 5-50 μl , pipette tips.
10. Automatic pipette 1 ml, pipette tips.
11. 0.05M phosphate buffered saline, pH7.4 (PBS)
12. Timer
13. Vortex mixer
14. Dose calibrator set for ^{125}I .
15. Tec-Control Chromatography 150-771 strips (Biodex)
16. 70% acetone/30% water mixture
17. PhosphorImager or TLC scanner (optional).

2.3. Indirect radioiodination of an antibody using N-succinimidyl para-iodobenzoate

N-succinimidyl 4-(trimethylstannyl)benzoate can be synthesized according to method described by Kozirowski et al (*15*).

1. Freeze-dried antibody
2. ^{125}I stock solution (GE Healthcare or PerkinElmer)
3. Siliconized Eppendorf tubes (1.7 ml)
4. 0.07 M sodium borate, pH 9.3

5. N-succinimidyl 4-(trimethylstannyl)benzoate (ATE)
6. Chloramine-T trihydrate, sodium chloro-(4-methylphenyl)sulfonylzanide (Sigma or Merck)
7. Sodium metabisulfite, $\text{Na}_2\text{S}_2\text{O}_5$ (Sigma or Merck)
8. 0.1% aqueous solution of acetic acid
9. 5% acetic acid in methanol
10. Milli-Q/ELGA water
11. Vortex mixer
12. Timer
13. NAP-5 column (GE Healthcare)
14. 0.05M phosphate buffered saline, pH7.4 (PBS)
15. Automatic pipette 1 ml, pipette tips
16. Automatic pipette 2-50 μl , pipette tips
17. Tec-Control Chromatography 150-771 strips (Biodex)
18. 70% acetone/30% water mixture
19. TLC tank
20. Dose calibrator set for ^{125}I .
21. PhosphorImager or TLC scanner (optional).

2.4. Labelling of an antibody with ^{111}In using CHX-A''DTPA

1. Ion-exchange resin Chelex 100 in sodium form (Sigma)
2. Disposable 0.4 μm filters
3. Disposable syringes
4. Disposable polypropylene 20 ml vials.
5. Freeze-dried antibody

6. Siliconized Eppendorf tubes (1.7 ml)
7. 0.07 M sodium borate, pH 9.3, stored over Chelex 100
8. 0.2 M ammonium acetate, pH 5.5, stored over Chelex 100
9. CHX-A''DTPA, N-[(R)-2-Amino-3-(p-isothiocyanato-phenyl) propyl]-trans-(S,S)-
10. cyclohexane-1,2-diamine-N,N,N',N'',N'''-pentaacetic acid, (Macrocyclics)
11. Vortex mixer
12. Heating block providing 38°C.
13. NAP-5 column (GE Healthcare)
14. Automatic pipette 1 ml, pipette tips
15. Automatic pipette 2-50 µl, pipette tips
16. ¹¹¹In chloride in 0.05 M hydrochloric acid for labelling of antibodies (Covidien)
17. Tec-Control Chromatography 150-771 strips
18. 0.2 M citric acid
19. PhosphorImager or TLC scanner (optional).

2.5. Determination of immunoreactive fraction

1. Cells, adherent or in suspension, 4×10^7 cells
2. Radiolabelled antibody
3. Non-labelled antibody
4. 0.05M phosphate buffered saline, pH7.4 (PBS)
5. Polypropylene centrifuge tubes, 15 ml
6. Siliconized Eppendorff tubes
7. Pipette controller (e.g. Pipetboy)
8. Glass or plastic pipettes, 10 ml

9. Set of automatic pipettes 1 μ l - 1 ml, pipette tips
10. Cell scraper for adherent cells
11. Vortex mixer
12. Cell counter
13. Centrifuge (at least 5000 g, with timer)
14. Gamma-counter

2.6. Determination of dissociation constant/saturation assay

1. Cells, adherent
2. Radiolabelled antibody
3. Non-labelled antibody
4. 0.05M phosphate buffered saline, pH7.4 (PBS)
5. Complete cultivation medium for the designated adherent cell line
6. Trypsin-EDTA, 0.25% trypsin, 0.02% EDTA in buffer or other appropriate buffer
for cell detachment
7. Polypropylene centrifuge tubes, 15 ml
8. Disposable cell dishes or 24-well cell plates
9. Siliconized Eppendorf tubes
10. Disposable test tubes
11. Vials for cell counting
12. Pipette controller (e.g. Pipetboy)
13. Disposable plastic pipettes (10 ml)
14. Set of automatic pipettes 1 μ l-1 ml, pipette tips
15. Vortex mixer

16. Cell counter

17. Gamma-counter

2.7. LigandTracer

1. Adherent cells, expressing at least 30,000 – 50,000 antigen copies per cell
2. Circular cell dish with 87mm outer bottom diameter (e.g. Nunclon™ cat. No. 150350 or 172958 on www.nuncbrand.com)
3. Cell culture medium, approximately 50 ml
4. Radiolabeled antibody, typically 3-30 µg (depends on the apparent affinity)
5. LigandTracer instrument suitable for the selected radiolabel (¹²⁵I: LigandTracer Grey, PET/SPECT radionuclides: LigandTracer Yellow)

3. Methods

3.1. Purification of an antibody

Purification of antibodies is essential for all conjugation labelling techniques. Most commonly, a conjugation of a chelator or a linker is directed to an amino group of a protein. Very often, antibody preparations contain free amino acids. The presence of free amino acids may interfere with amino-directed coupling.

1. Pre-equilibrate the NAP-5 column with Milli-Q water by passing 10 ml of Milli-Q water through the column.
2. Load 0.5 ml of the antibody solution on the column.
3. Collect and discard first 0.5 ml of eluate.
4. Add 1 ml of Milli-Q water to a column.
5. Collect eluate into an Eppendorf tube.
6. Freeze the eluate at -18 °C for at least 3 hours

7. Freeze-dry the eluate overnight

3.2. Direct radioiodination of an antibody with ^{125}I using Chloramine-T

Important! The work should be performed in a well-ventilated fume-hood. Contamination control should be performed after labelling.

1. From the freeze-dried antibody, prepare a solution in PBS containing 2 mg/ml.
2. Prepare Eppendorf tubes containing 1-1.5 mg of Chloramine-T and sodium metabisulfite
3. Before starting of labelling, pre-equilibrate a NAP-5 column with PBS, by passing at least 10 ml of the buffer through the column
4. Place 10 μl ^{125}I stock solution into an Eppendorf tube (**Note.** Up to 20 μl of ^{125}I stock solution can be used according to this protocol)
5. Add 20 μl antibody solution (40 μg) to the ^{125}I solution.
6. Add 40 μl PBS to the mixture of ^{125}I and the antibody solution.
7. Prepare immediately before labelling a Chloramine-T solution in PBS (1 mg/ml) and sodium metabisulfite solution (2 mg/ml in PBS)
8. Add 15 μl of Chloramine-T solution in PBS to the mixture of ^{125}I and antibody. Vortex carefully and incubate the mixture for 60 sec.
9. Add 15 μl sodium metabisulfite solution to the reaction mixture, vortex the mixture carefully. Calculate the mixture volume, X μl .
10. Load the reaction mixture on the NAP-5 column. Let it pass through the upper filter. Then add (500-X) μl PBS and let it pass through the upper filter.
11. Collect the eluate as a void volume fraction.
12. Add 1 ml PBS. Collect the eluate a high molecular weight fraction containing the labelled antibody.
13. Add 1 ml PBS. Collect the eluate a low molecular weight fraction.

14. Cap the column, start with the lower end to reduce the risk of contamination. Measure activity of empty reaction mixture vial, the high molecular weight fraction, the low molecular weight fraction and the column to calculate yield according to formula

$$Yield = [activity\ of\ high\ molecular\ weight\ fraction] / [sum\ of\ all\ measured\ activities]$$

15. Take 1 µl sample of the high molecular weight fraction and place on Tec-Control

Chromatography 150-771 strip. Elute the strip with the 70% acetone/30% water mixture

16. Evaluate purity of the conjugate. The radiolabelled antibody would stay at the application point, while free ¹²⁵I would migrate with the solvent front. This might be done quantitatively using PhosphorImager or TLC scanner. Alternatively one can cut the Tec-Control Chromatography 150-771 in the middle. After measurement of background (B), measure radioactivity of half with the application point (A) and half with the solvent front (F). Calculate the purity according to formula

$$P(\%) = (A-B)*100/(A+F-2*B)$$

17. ¹²⁵I-antibody can typically be stored frozen at -20 °C for a few days

3.3. Indirect radioiodination of an antibody using N-succinimidyl para-iodobenzoate

Important! The work should be performed in a well-ventilated fume-hood. Contamination control should be performed after labelling.

1. Prepare an Eppendorf tube, containing approx. 1 mg antibody.
2. Prepare Eppendorf tubes containing 1-1.5 mg of Chloramine-T and sodium metabisulfite.
3. Prepare an Eppendorf tube containing 0.5-1 mg of N-succinimidyl 4-(trimethylstannyl) benzoate (ATE)

4. Immediately before labelling, dissolve the antibody in 0.07 M sodium borate, pH 9.3, to a concentration of 3 mg/ml;
5. Immediately before labelling, dissolve Chloramine-T in Milli-Q/ELGA water to a concentration of 4 mg/ml;
6. Immediately before labelling, dissolve sodium metabisulfite in Milli-Q water to a concentration of 8 mg/ml;
7. Immediately before labelling, dissolve ATE in 5% acetic acid in methanol to a concentration of 1 mg/ml;
8. Place 4-10 μl ^{125}I stock solution into an Eppendorf tube.
9. Add 10 μl of 0.1% aq. solution of acetic acid to ^{125}I .
10. Add 5 μl ATE-solution to the mixture. Vortex carefully.
11. Add 10 μl Chloramine-T solution to the mixture. Vortex carefully!
12. Incubate the mixture for 5 min at room temperature.
13. Add 10 μl sodium metabisulfite solution to the mixture. Vortex carefully.
14. Add 100 μl of the antibody solution to the mixture. Vortex carefully.
15. Incubate the mixture 60 min at room temperature. Calculate the mixture volume, X μl .
16. During incubation, pre-equilibrate a NAP-5 column with PBS (at least 10 ml).
17. Load the reaction mixture on the NAP-5 column and let it pass through the upper filter.
18. Add (500-X) μl PBS and let it pass through the upper filter
19. Collect the eluate as a void volume.
20. Add 1 ml PBS. Collect the eluate a high molecular weight fraction containing the labelled antibody.
21. Add 1 ml PBS. Collect the eluate a low molecular weight fraction.

22. Cap the column, start with the lower end to reduce the risk of contamination. Measure activity of empty reaction mixture vial, the high molecular weight fraction, the low molecular weight fraction and the column to calculate yield according to formula
$$\text{Yield} = [\text{activity of high molecular weight fraction}] / [\text{sum of all measured activities}]$$
23. Take 1 μl sample of the high molecular weight fraction and place on Tec-Control Chromatography 150-771 strip. Elute the strip with the 70% acetone/30% water mixture
24. Evaluate purity of the conjugate using the Tec-Control strips, as it has been described in 3.2.16. The radiolabeled antibody would stay at the application point, while free ^{125}I and ^{125}I -iodobenzoic acid would migrate with the solvent front.

3.4. Labelling of antibody with ^{111}In using CHX-A''DTPA

3.4.1. Preparation of metal-free buffers (see Note 2)

1. Prepare 0.07 M sodium borate, pH 9.3, and 0.2 M ammonium acetate, pH 5.5, using a high-quality water and p.a. reagents.
2. Add Chelex 100 (10 g per litre of buffer), mix carefully and let stay overnight.
3. Immediately before use, filter the buffer through a 0.4 μm filter into a disposable polypropylene vial. Use first 5 ml to rinse vials.

3.4.2. Conjugation of CHX-A''DTPA to an antibody

1. Collect in an Eppendorf tube ~ 1.4 mg freeze-dried antibody. Note the exact weight.
2. Calculate required amount of CHX-A''DTPA. For a coupling of four chelator per an antibody molecule, 0.0188 μg CHX-A''DTPA per 1 μg of antibody is required.
3. Take in an Eppendorf tube 0.7-1.2 mg CHX-A''DTPA. Note the exact weight.

4. Dissolve CHX-A''DTPA in 0.07 M sodium borate, pH 9.3, to obtain a final concentration of 1mg/ml. Use sonication if reagents dissolves slowly.
5. Add a calculated amount of CHX-A''DTPA in 0.07 M sodium borate, pH 9.3, to the antibody powder.
6. Add 200 μ l of 0.07 M sodium borate, pH 9.3. Vortex the mixture carefully! Calculate the volume of the solution, X μ l.
7. Incubate the reaction mixture for at least 4 h (preferably overnight) at 38 °C.
8. Pre-equilibrate a NAP-5 column with 0.2 M ammonium acetate, pH 5.5, stored over Chelex 100, by passing at least 10 ml of the buffer through the column.
9. Load the reaction mixture on the column. Let it pass through the upper filter.
10. Add (500-X) μ l of 0.2 M ammonium acetate, pH 5.5.
11. Collect and discard the eluate.
12. Add 900 μ l of 0.2 M ammonium acetate, pH 5.5, to the column, collect the eluate.
13. You can consider that all your antibody is eluted in 900 μ l. Calculate the antibody concentration.
14. Divide the eluate, containing CHX-A''DTPA-cetuximab into aliquots containing 100 μ g of antibody. The aliquots can be stored frozen at -20 °C.

3.4.3. Labelling of CHX-A''DTPA cetuximab with ¹¹¹In

1. Calculate a volume of ¹¹¹In stock solution required for approx. 10 MBq.
2. Add a required volume of ¹¹¹In to an aliquot of CHX-A''DTPA-antibody conjugate in 0.2 M ammonium acetate, pH 5.5 (100 μ g)
3. Vortex the mixture carefully and incubate at room temperature for 1 hour.

4. Take 1 μl sample and place on Tec-Control Chromatography 150-771 strip. Elute the strip with 0.2 M citric acid.
5. Evaluate purity of the conjugate as described in 3.2.16. The radiolabelled antibody would stay at the application point, while free ^{111}In would migrate with the solvent front.
6. If the purity of ^{111}In – labelled antibody is over 95%, dilute the mixture to PBS to 1 ml. If the purity is below 95%, purify the conjugate using NAP-5 column according to manufacturer's instructions.
7. ^{111}In –labelled antibody can be stored frozen at -20°C for a few days.

3.5. Determination of immunoreactive fraction

This protocol was established in our laboratories based on the methodology described by Lindmo and co-workers (*14*). We refer readers interested in the theoretical background of the method to that publication. See **Notes 3-5**.

1. Calculate a volume of a labelled antibody to prepare 15 ml solution with concentration of $20 \times K_D$ (where K_D is an apparent dissociation constant of non-labelled antibody at equilibrium). If K_D is not known, assume that $K_D = 30 \text{ nM}$.
2. Calculate an amount of non-labelled antibody to prepare 1.1 ml solution with concentration $1000 \times K_D$.
3. Adherent cells: Scrape cells and re-suspend them in PBS by pipetting
4. Count cells to assess cell concentration per ml.
5. Prepare four Eppendorf tubes, each with a cell pellet containing 10^7 cells in 1 ml by gentle centrifugation. Start with 15 ml centrifuge tubes if needed.
6. Put cell samples on ice or move them to a cold room (4°C).

7. Prepare a 1.1 ml aliquot of the non-labelled antibody in a polypropylene centrifuge tube, mark it, vortex gently.
8. Add 1 ml of the non-labelled antibody solution to one of the Eppendorf tubes with a cell pellet, re-suspend the cells.
9. Add 1 ml PBS to other three tubes with cell pellets, re-suspend the cells.
10. Prepare from each Eppendorf tube a series of 5-6 cell samples (0.5 ml) by consecutive dilutions by 1:2 in PBS, leave 0.5 ml cell suspension in the last series sample, mark samples.
11. Prepare a 15 ml aliquot of the labelled antibody in a polypropylene centrifuge tube, mark the tube, vortex gently.
12. Add 0.5 ml of the labelled antibody to each vial containing cells.
13. Incubate the cells with the radiolabelled antibody at 4°C for at least 4 h.
14. Form cell pellets by centrifugation (at least 5000 g for 5 min);
15. Take 0.5 ml of supernatant from each Eppendorf tube to empty Eppendorf tube, mark the tubes.
16. Measure radioactivity content in samples.
17. Measure the background radioactivity of the sample holders used for radioactivity measurement.
18. Subtract the background radioactivity to obtain a background-corrected value for each supernatant and each pellet.
19. Calculate cell associated radioactivity $A(\text{cells})$ according to

$$A(\text{cells}) = \frac{A(\text{pellet} + \text{supernatant})}{A(\text{pellet} + \text{supernatant}) + A(\text{supernatant})}$$
20. For each data point, subtract unspecific binding ($A(\text{cells})$ values for samples incubated with non-labelled antibody)
21. Calculate for each data point

$$Y = \frac{A(\text{pellet} + \text{supernatant}) + A(\text{supernatant})}{A(\text{cells})}$$

22. Calculate to each data point inverse cell concentration

$$X = \frac{ml}{lg(\text{cells in sample})}$$

23. Plot a graph of ratio of total added radioactivity to cell bound radioactivity as a function of inverse cell concentration (ml/cells) and extrapolate the line to interception with *Y* axes ($X=0$) (**Figure 2**). We recommend using appropriate program for calculation (e.g. GraphPad Prizm, GraphPad Software Inc).

24. Calculate Immunoreactive fraction as **IRF** = 100% * 1/Y($X=0$)

[Fig 2 near here]

3.6. Determination of dissociation constant/saturation assay

1. Prepare a set of four cell culture dishes with adherent antigen-expressing cell per concentration used, typically 32 – 48 dishes (triplicate plus one cell culture dish for determination of nonspecific binding by antigen blocking) . Seed cells in advance, taking in account cells character (doubling time, receptor expression, time to stable attachment, etc)
2. Calculate the labelled antibody concentrations (8-12 data points) starting from $0.2 \times K_D$ to $20 \times K_D$ (where K_D is an apparent dissociation constant of non-labelled antibody at equilibrium). If K_D value is unknown, we recommend a

concentration range of 200pM-100nM. Remember that actual concentration added to cell samples will be twice lower! See **Notes 6 and 7**.

3. Calculate dilution conditions for designed antibody concentrations, typically 1:3 (at least 3 ml solution for every concentration).
4. Calculate the amount of non-labelled protein required to prepare 7 ml solution with concentration $60 \times K_D$.
5. Prepare solution of non-labelled antibody in PBS (in a polypropylene centrifuge tube, 15 ml), mark, vortex gently.
6. Prepare solution of the labelled antibody in PBS (in a polypropylene centrifuge tube, 15 ml), mark the tube, vortex gently.
7. Prepare a dilution series of labelled antibody solutions according to calculations using PBS, mark vials, vortex.
8. Wash cells with fresh media.
9. Put culture dishes with cell on ice or move them to cold room (4°C)
10. Add 500 μ l of non-labelled antibody solution to one of cell dishes for every data point, mark. Antigens in this dish will be close to saturated, and the majority of antibody binding will be unspecific. This dish will be designated as an unspecific binding control sample.
11. Add 500 μ l PBS to all other cell culture dishes.
12. Add 500 μ l of the labelled antibody solution with the lowest concentration to cell culture dishes (triplicate samples plus an unspecific binding control sample), mark dishes.
13. Take one standard sample (500 μ l) to a test tube, mark tube.
14. Repeat these two (12-13) steps for every concentrations
15. Incubate cell samples at 4°C for 4 h

16. After incubation, aspirate the radioactive solution, wash cells with fresh PBS, detach cells with 0.5 ml trypsin-EDTA solution (or other reagent), add 1 ml PBS, re-suspend cells, take 0.5 ml for cell counting, collect the rest of cell suspension to test tubes for measurement, mark test tubes with radioactive samples.
17. Count cells in all samples.
18. Measure radioactivity in all cell samples and the concentration standards.
19. Measure background radioactivity in the sample holders.
20. Subtract background radioactivity for each sample to obtain background-corrected values.
21. Calculate the real added radiolabelled antibody concentrations for each data point assuming that the highest concentration as the most reliable one (minimum losses due to protein absorption).
22. Calculate measured radioactivity for the highest concentration of added radiolabelled antibody as counts per minute (CPM) per pmol
23. Calculate bound radioactivity per cell for every sample (e.g. CPM/ 10^6 cells)
24. Subtract unspecifically bound radioactivity (radioactivity of the unspecific binding control sample for this data point) and obtain specifically bound radioactivity for every data point
25. Calculate specifically bound radioactivity as pmol/ 10^6 cells for every sample
26. Plot a graph specifically bound radioactivity per cell (pmol/ 10^6 cells) vs. concentration (pM). Determine B_{\max} (maximum number of binding sites per cell) and calculate K_D as a concentration of radiolabelled antibody causing binding equal to $B_{\max}/2$ (**Figure 3**). We recommend using an appropriate software (e.g. GraphPad Prism, GraphPad Software Inc). See **Notes 8 and 9**.

[Fig 3 near here]

3.7. Determination of dissociation constant using LigandTracer.

LigandTracer technology has been described in a series of papers and the technical details of how to successfully conduct a real-time binding assay on living cells has been discussed elsewhere (*16, 17*). Read the instrument instruction booklet prior to starting – standard operating procedures for maintaining and operating the instrument are clearly described there.

In brief, LigandTracer technology relies on a circular cell dish containing adherent cell in a limited portions, where the dish is placed on a tilted, slowly rotating support (**Figure 4**). The radioactivity detector is placed over the elevated portion of the dish and registers the additional radioactivity brought under the detector by the cells. Hence, if the radiolabelled antibody binds to the cells, the detector will register an increased signal when the cells are below the detector than when the cells are out-of-view. Since the dish rotates continuously, approximately one data point per minute is collected, making it possible to follow the progress of the interaction over time.

[Fig 4 near here]

3.7.1. Cell dish preparations

1. Take an 87 mm cell dish and place it tilted on some object (a cell dish lid is usually fine).
2. Carefully dispense 1-2 ml of cell culture medium containing ~1 million adherent cells into the lower part.
3. Place in an incubator (still tilted) and let the cells attach firmly. This typically takes 8 hours, but it is strongly dependant on the cell line.

4. When the cells have attached, aspirate the medium, place the dish horizontally, add ~10 ml of cell culture medium, and culture the cells for at least 24 h before using the dish.

3.7.1. Antibody binding measurement

1. Place the dish in the appropriate LigandTracer device and keep 3 ml of cell culture medium in the dish. Most LigandTracer assays are conducted at room temperature, but it is possible to use the device at reduced temperature (+4-8°C) if deemed necessary.
2. Start the device and collect a baseline using the default settings during 3-10 minutes.
3. Stop the device and add a small aliquot of labelled antibody to the dish. Add one µg of antibody to the three ml liquid already present in the dish, this corresponds to approximately two nM. Start the device and wait 120-180 minutes.
4. Inspect the shape of the binding curve and compare it to **Figure 5**.
 - a. If the first incubation step resulted in a curve approaching equilibrium, stop the instrument and add two µg antibody to the dish
 - b. If the first incubation step resulted in a linear curve of increasing signal, stop the instrument and add nine µg antibody to the dish
 - c. If no linear or curvilinear signal increase is seen, add nine µg antibody to the dish
5. Start the device and wait 120-180 minutes.
6. Inspect the shape of the binding curve and compare it to **Figure 5**.
 - a. If the first and/or the second incubation step resulted in a curve approaching equilibrium, stop the instrument and prepare for retention (step 9 below)
 - b. If the second incubation step resulted in a curve approaching equilibrium, stop the instrument and prepare for retention (step 9 below)

- c. If the first and the second incubation steps resulted in a linear increase of signal, stop the instrument and add 20 µg antibody to the dish
 - d. If no linear or curvilinear signal increase is seen, add 20 µg antibody to the dish
7. Start the device and wait 120-180 minutes.
8. Inspect the shape of the binding curve and compare it to **Figure 5**.
 - a. If there is a visible binding signal, prepare for retention (step 9 below)
 - b. If there is no visible binding signal, stop the measurement and conclude that no binding can be detected.
9. Retention measurement: Stop the instrument and aspirate the liquid in the cell dish. Add 3 ml of fresh cell culture medium. It is typically not required to include any wash steps. Restart the instrument and let it collect data for 3-15 hours.

[Fig 5 near here]

3.7.1. Data analysis

Ocular analysis of the binding trace is often sufficient for a qualitative statement on if the antibody has the ability to bind the antigen. The most important feature to look for is a binding signal collected during incubation that approaches equilibrium. (**Figure 5 A and B**). Such a curve shape indicates that the antibody binds to a finite number of antigen molecules on the cells. Exclusively linear uptake curves (**Figure 5 C**) are inconclusive, but indicate that either higher concentration (or more time) is needed or that the interaction is unspecific (i.e. having an infinite or at least very large number of binding sites on the cells). Lack of signal (**Figure 5 D**), or square pulses (**Figure 5 D**), is usually a sign of inability for the antibody to bind.

The retention measurement which is conducted after replacing the antibody with fresh cell culture medium reveals how long time the antibody stays in complex with the antigen. Most antibodies stay bound with their antigen for several hours, resulting in a slowly decreasing signal (**Figure 6 A**)

The affinity and the binding kinetics can be resolved through fitting the time-resolved interaction model to the collected data in specialized software, e.g. TraceDrawer. Such an analysis can produce precise and accurate estimates on both binding strength (affinity), and time to equilibrium (binding kinetics), and may even reveal how many parallel processes that are hidden in one and the same binding trace (Interaction Map). Detailed discussions on kinetic analysis are beyond the scope of this publication, but plenty of material has been published elsewhere (*18, 19*). See **Note 10**.

[Fig 6 near here]

4. Notes

Note 1. Radionuclides emit ionizing radiation, which is potentially damaging for workers. During work, the Radiation Safety guidelines set by institutions and the national nuclear regulatory authorities must be followed strictly and meticulously. Protective equipment, personal dosimeters and radiation survey monitors are required when handling any radioactive materials.

Note 2. CHX-A''DTPA has no particular selectivity for radionuclides and reacts with a broad range of transitional metals. Metal contaminations may saturate the chelator and prevent binding of radionuclides. All solution should be prepared using high quality (Milli-Q or ELGA) water. Buffers should be purified from metal contamination using Chelex 100 resin. Colourless polypropylene Eppendorf tubes and pipette tips contain usually low level of metal

impurities, and might be used directly. However, care should be taken to prevent them from dust contamination, as dust might contain metals. For some preparations with very high specific activity, reaction tubes and pipette tips might additionally be treated as described by Wadas and Anderson (20).

Note 3. Receptor expression on the selected cell line can be a limiting factor in case of low expression level or high K_D value due to receptor depletion. In the sample with highest cell concentration, antigens have to be in a large excess over antibody.

Note 4. Time to equilibrium in the antibody/antigen interaction can be another limiting factor. In the case of slow binding kinetics, incubation time should be prolonged.

Note 5. In the case when cell number is not a limiting factor and receptor expression is high, estimation of Immunoreactive fraction can be done using one data point with cell pellets of $1-2 \times 10^7$ cells/pellet. In such conditions $X \rightarrow 0$ (21). The Immunoreactive fraction can be calculated as

$$IRF = \frac{A(\text{pellet} + \text{supernatant}) * 100\%}{A(\text{pellet} + \text{supernatant}) + A(\text{supernatant})}$$

Note 6. Radioactivity in solution with the lowest and the highest concentrations added to the cells have to be measurable using gamma-counter. If radioactivity in solutions for high concentrations will be too high (causing problems with the dead time of the counter) samples can be divided in several test tubes. Do not forget to collect pipette tips for radioactivity measurements.

Note 7. The accuracy of the method depends on accurate estimation of the antibody concentrations used in the experiment. Take in account that in consecutive dilutions real protein concentrations can be lower than calculated ones due to absorbance to plastic.

Therefore it is strongly recommended to take a standard sample to every added concentration and re-calculate the real concentrations based on radioactivity measurements.

Note 8. Internalization of radiolabelled antibodies may influence appreciable the measurement result leading to overestimation of the bound radioactivity in the case of residualizing radiometal labels (due to intracellular trapping of internalized antibody) and underestimation of bound activity in the case of radioiodine labels (due to leakage of radiocatabolites). **For this reason, the assay must be performed at 4°C or on ice, when internalization is inhibited.**

Note 9. Remember that the time to equilibrium depends on concentration, on-rate and off-rate of the antibody-antigen interaction. In the case of strongly binding antibody (and in particular low off-rate), time of equilibrium might be equal to several hours or even days. Too short incubation would cause a serious underestimate of the affinity. For this reason, it is desirable to evaluate a binding kinetics of an antibody **at lowest concentration** before determination of K_D and select incubation time when the plateau of uptake is achieved. The use of LigandTracer is free from this limitation.

Note 10. If the cells detach during the LigandTracer measurement, inaccurate data will be collected. The detection limit of LigandTracer method depends on the cellular system, but is typically 30000-50000 receptors per cell.

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Figure captions

Figure 1. Labelling of antibodies with ^{125}I by oxidative radioiodination using Chloramine-T (A), indirect radioiodination using N-succinimidyl 4-trimethylstannylbenzoate (B)- Conjugation of CHX-A''-DTPA for labelling with ^{111}In (C).

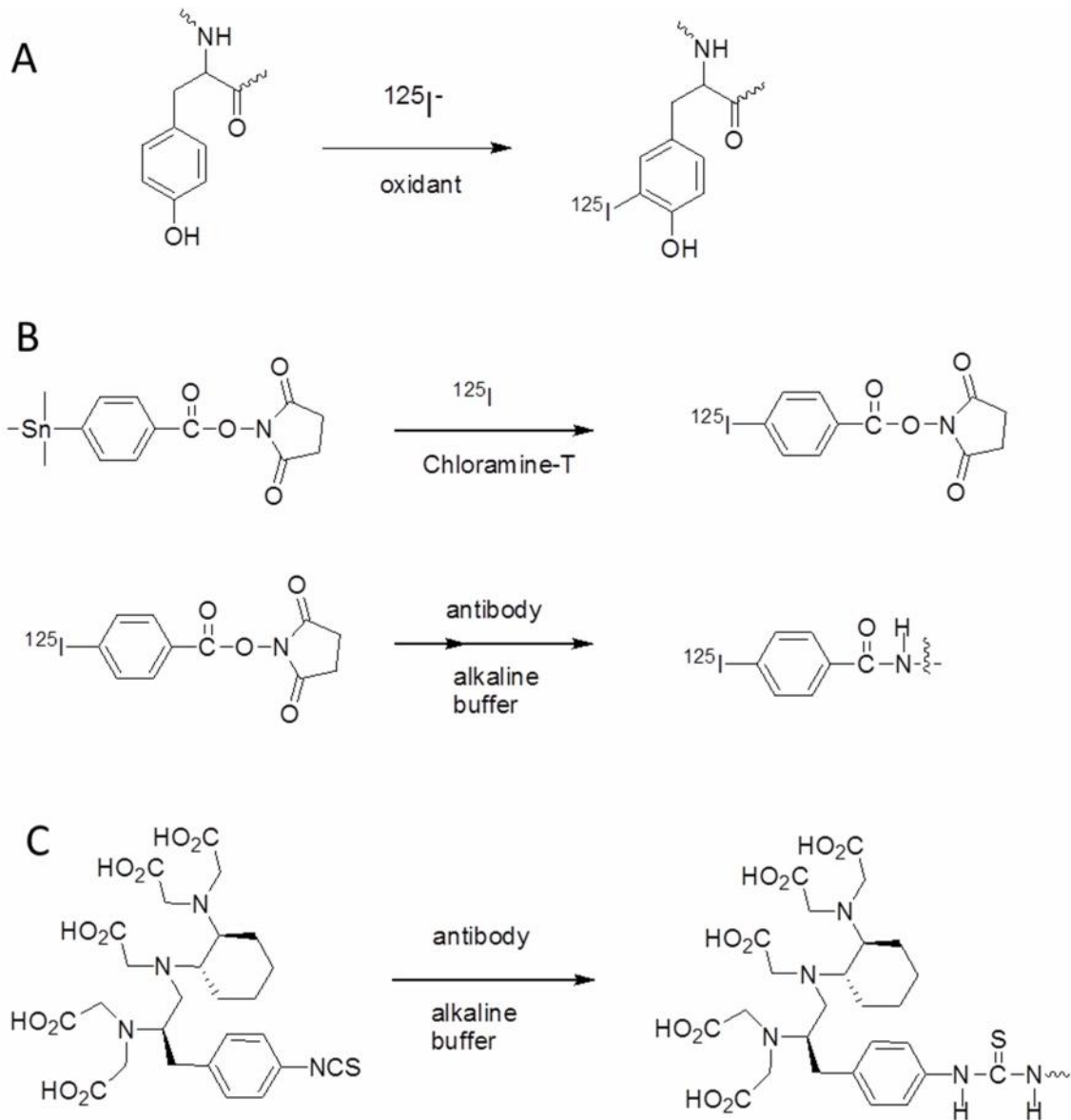


Figure 2. Ratio of total added radioactivity to cell bound radioactivity as a function of inverse cell concentration. The immunoreactive fraction was calculated as $1/ Y(X=0)$

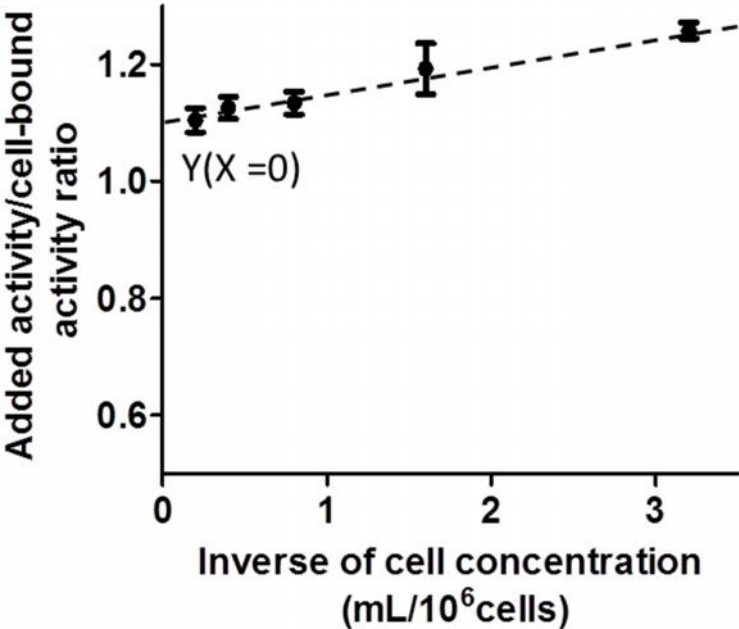


Figure 3. Graphical determination of K_D (apparent dissociation constant of non-labelled antibody at equilibrium) for a radiolabelled antibody.

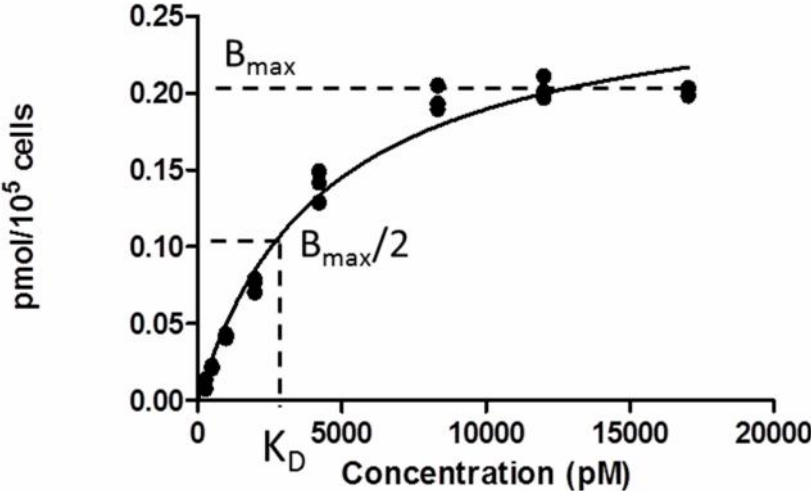


Figure 4. Schematic illustration of LigandTracer technology.

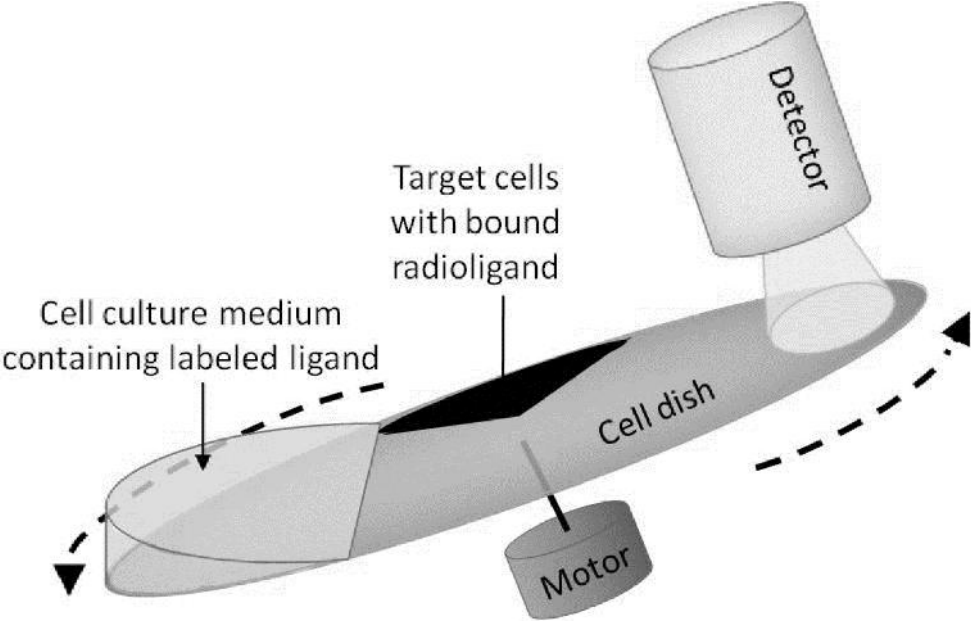


Figure 5. Illustrative examples of two incubation steps of increasing antibody concentration in a LigandTracer assay. A: Binding curve clearly approaching equilibrium at both concentrations is a strong proof for specific interaction. B: First a linear signal increase, followed by the binding curve approaching equilibrium is a strong proof for specific interaction. C: Linear signal increases at all tested concentrations can have multiple causes, including too low antibody concentration and unspecific interaction, and is hence inconclusive regarding binding properties. D: No or very small signal changes throughout the assay is usually indicative of antibody inability to bind antigens, but may also be due to insufficient number of cells. E: Square pulse like binding traces is usually indicative of antibody inability to bind antigens but can have multiple causes, including weak antibody interaction.

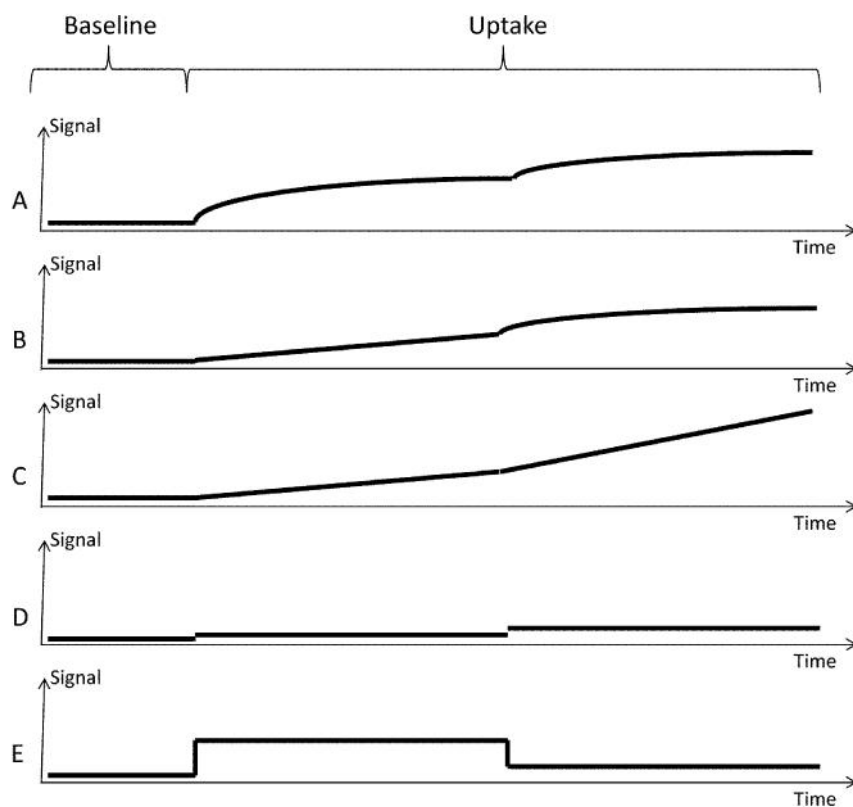


Figure 6. Antibody trastuzumab (labelled with ¹²⁵I using the CAT protocol) interacting with HER2 expressed on SKOV3 cells, as measured using LigandTracer Grey.

