

## Fluorescence Staining of Cell Surface HER2

### INTRODUCTION

Fluorescence staining is a powerful analytical tool used to determine the expression and distribution pattern of a particular protein in cells and tissues. With a fluorescence microscope, it is possible to follow changes in localization of a protein after external stimuli. With flow cytometry, analysis of several protein markers can be performed simultaneously using different types of non overlapping fluorescent dyes, performing population dynamics studies.

The Anti-HER2 Affibody<sup>®</sup> molecule is a specific affinity ligand that can advantageously be used for fluorescence staining of the tumour marker HER2 on cell surfaces as well as on frozen tissue sections. The Anti-HER2 Affibody<sup>®</sup> molecule is available as a biotin conjugated reagent for staining with fluorescence conjugated streptavidin or as a fluorescein conjugated and convenient one step reagent. The Anti-HER2 Affibody<sup>®</sup> molecule is also available as an unconjugated reagent that is easily coupled to any thiol-activated fluorescent dye.

### RESULTS FLUORESCENCE STAINING OF CELLS

The human mammary gland cell line SK-BR3 expresses high levels of HER2 and this cell line was used to demonstrate Affibody<sup>®</sup> fluorescence staining and to compare three different staining reagents for cells; fluorescein conjugated; biotin conjugated and Oregon Green<sup>®</sup> labeled Affibody<sup>®</sup> molecules. The SK-BR3 cells were stained for 30 minutes at a concentration of 1-5 µg Affibody<sup>®</sup> molecule/ml.

As seen in figure 1, there is bright membrane staining with all three reagents. As both the fluorescein conjugated and Oregon Green<sup>®</sup> labeled Affibody<sup>®</sup> molecule function as one step reagents, the staining procedure was completed in only 30 minutes.

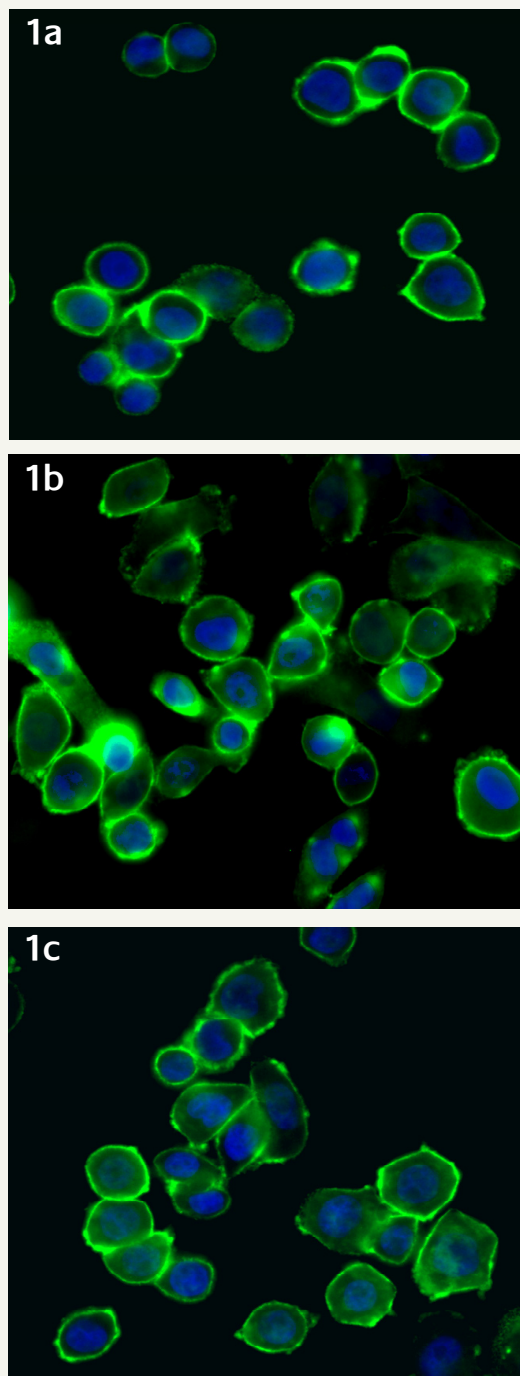


Fig. 1. SK-BR3 cell were stained with a) fluorescein conjugated Anti-HER2 Affibody<sup>®</sup> molecule, b) biotin conjugated Anti-HER2 Affibody<sup>®</sup> molecule and Streptavidin-Alexa<sup>®</sup> 488 and c) Oregon Green<sup>®</sup> conjugated Anti-HER2 Affibody<sup>®</sup> molecule. The staining was localized to the membrane of the HER2 expressing human mammary gland cells. Nuclei were counter stained with DAPI (blue fluorescence).

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### RESULTS FLUORESCENCE STAINING OF FROZEN TISSUE SECTIONS

Frozen tissue sections were obtained from snap frozen SK-OV-3 xenografts. The sections were stained with Oregon Green<sup>®</sup> conjugated Anti-HER2 Affibody<sup>®</sup> molecule for 30 minutes at a concentration of 2  $\mu\text{g}$  Affibody<sup>®</sup> molecule/ml. The resulting microscope image shows brightly stained SK-OV-3 cells inside the tumor whereas the connective tissue surrounding and traversing the tumor cells remained negative.

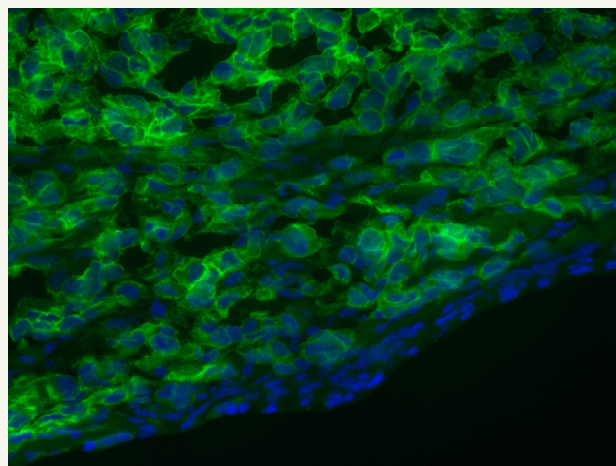


Fig. 2. Sections of human ovarian cancer cell line SK-OV-3 xenografts were stained with Oregon Green<sup>®</sup> conjugated Anti-HER2 Affibody<sup>®</sup> molecule. The staining was localized to the membrane of the HER2 expressing SK-OV-3 cells. Nuclei were counterstained with DAPI (blue fluorescence).

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### MATERIALS AND BUFFERS REQUIRED

**Staining reagents:** Anti-HER2 Affibody<sup>®</sup> molecule, unconjugated (Affibody cat no 10.0817.01.0005). Anti-HER2 Affibody<sup>®</sup> molecule, biotin conjugated (Affibody cat no 10.0817.02.0005). Anti-HER2 Affibody<sup>®</sup> molecule, fluorescein conjugated (Affibody cat no 10.0817.03.0005).

**Multi-well slides:** (Histolab Products cat no 06278)

**Round-bottom tubes:** 5 ml (Falcon cat no 352063)

**PBS:** 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4

**DAPI:** (Molecular Probes cat no D1306)

**Anti-fading reagent:** (Vector Laboratories cat no H-1000)

**Fluorescent conjugation:** Streptavidin ALEXA (Molecular Probes cat no S11223)

### PREPARATION OF CELLS FOR STAINING ON MULTI-WELL SLIDES

1. Adherent cells: Grow cells in a suitable medium over night on multi-well slides to obtain flattened morphology of adherent cells. A confluence of less than 70% facilitates microscopy examination.
2. Next day, gently remove medium and fix the cells with 3% formaldehyde in PBS for at least 10 minutes in room temperature.
3. Wash the slides gently 2 times with PBS.

**Note:** Cells in suspension are stained as described below for flow cytometry. Suspension cells can then be added to multi-well slides and fixed with 3% formaldehyde.

### STAINING ON MULTI-WELL SLIDES

1. If necessary, block the slides with 1% Bovine Serum Albumin or 1% Fetal Calf Serum in PBS for 15 minutes by adding one droplet (25-50  $\mu$ l) blocking solution per well. **Note:** The need for blocking should be tested by the user. In general, Affibody<sup>®</sup> molecules show very little background staining. However, different cell lines and type of multi-well slides may behave differently and the need for blocking should therefore be tested by the user.
2. Remove the blocking solution gently. Wipe away any remaining buffer between the wells on the slide.
3. Add 25  $\mu$ l (for 8 multi-well slides) of the Affibody<sup>®</sup> molecule,

diluted in PBS. A concentration in the range of 0.1-5  $\mu$ g/ml Affibody<sup>®</sup> molecule is recommended. However, the optimal concentration should be determined by the user.

4. Incubate in a moist chamber for 30 minutes at room temperature.
5. Wash the slides gently 2 times with PBS.
6. If using fluorescein conjugated Affibody<sup>®</sup> molecule, proceed to 8. If using biotin conjugated Affibody<sup>®</sup> molecule, incubate with fluorescent conjugated streptavidin. The optimal streptavidin concentration should be determined by the user.
7. Wash the slides gently 2 times with PBS.
8. For contrast, counter stain the cell nuclei with DAPI, one droplet per well for 5-20 seconds and wash away DAPI excess with PBS.
9. Allow slides to dry completely, protected from light.
10. Mount with anti-fading reagent before examination in a UV microscope.

### STAINING OF FROZEN TISSUE SECTIONS

1. 4-6  $\mu$ m thick sections should be used.
2. Thaw slides for 10-15 minutes at room temperature.
3. Fix the tissue section with 3% formaldehyde in PBS for at least 10 minutes at room temperature.
4. Wash the slides gently in a cuvet with PBS 2 x 1 minute before staining.
5. Block slides with 1% FCS or 1% BSA in PBS for 15 minutes. Be sure that the blocking solution covers the tissue completely. If streptavidin is used, a biotin blocking step may be required.
6. Remove blocking solution and make sure that the surface is dry around the tissue section.
7. Add the conjugated Affibody<sup>®</sup> molecule, diluted in PBS. Make sure the added volume completely covers the tissue. A final concentration of 0.1-5  $\mu$ g/ml conjugated Affibody<sup>®</sup> molecule is recommended. The user is required to determine the optimal concentration.
8. Incubate in a dark, moist chamber for 45 minutes at room temperature.
9. Wash slides gently with PBS, approximately 2 x 5 minutes in a cuvet.
10. If using fluorescein conjugated Affibody<sup>®</sup> molecule, proceed to 12. If using biotin conjugated Affibody<sup>®</sup>

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molecule, incubate with fluorescent conjugated streptavidin. The optimal streptavidin concentration should be determined by the user.

11. Wash slides gently with PBS approximately 2 x 5 minutes in a cuvet (do not shake).
12. For contrast, stain the nuclei of cells with DAPI, one droplet per well for 5-20 seconds and wash away the excess with PBS.
13. Let the slides dry completely, protected from light.
14. Mount with anti-fading reagent.

## Conjugation of Affibody<sup>®</sup> Molecules

### MATERIALS AND BUFFERS REQUIRED

**PBS:** 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4

**Desalting column:** NAP5-column (GE Healthcare cat no 17-0853-01)

**Dithiothreitol (DTT) solution**

**Fluorescent dye:** Any thiol reactive fluorescent dye, e.g. Oregon Green<sup>®</sup> 488 maleimide (Molecular Probes O6034)

**Desalting spin columns:** (Pierce cat no 89849)

### INTRODUCTION

The Affibody<sup>®</sup> molecule is delivered with a unique C-terminal cysteine that is easily conjugated with any fluorescent dye of choice. Bright and strong staining has been obtained with the Oregon Green<sup>®</sup> dye, one of many dyes that are available as a thiol reactive derivative.

The Affibody<sup>®</sup> molecule is conjugated as described by the manufacturer. However, the Affibody<sup>®</sup> molecules are partially dimerized due to S-S bridges formed by the C-terminal cysteine and reduction of the Affibody<sup>®</sup> molecule immediately prior to conjugation is therefore an absolute necessity. A brief protocol for reduction of the C-terminal cysteine is found below.

### REDUCTION OF THE AFFIBODY<sup>®</sup> MOLECULE

1. Dissolve the lyophilized Affibody<sup>®</sup> molecule in PBS to obtain a final concentration of 1 mg/ml.
2. Add DTT to a final concentration of 20 mM at >pH 7.5.
3. Incubate at room temperature for 2 hours.
4. Remove excess DTT by passage through a NAP5-column. The Affibody<sup>®</sup> molecule will re-dimerize quickly and dialysis is therefore not recommended.
5. Immediately after step 4 above, add the conjugate at the recommended molar excess and follow the protocol from the fluorescent dye manufacturer.
6. After completed conjugation and dialysis, we strongly recommend an extra desalting step using protein desalting spin columns to remove all remaining free fluorescent dye.

### LIMITATIONS

Warranty: Affibody<sup>®</sup> products are warranted to meet stated product specifications and to confirm to label descriptions when used and stored properly. Unless otherwise stated, this warranty is limited to one year from date of sales for products used, handled and stored according to Affibody AB's instructions. Affibody AB's sole liability is limited to replacement of the product or refund of the purchase price. Affibody<sup>®</sup> products are supplied for research use only. They are not intended for medicinal, diagnostic or therapeutic use. Affibody<sup>®</sup> products may not be resold, modified for resale or used to manufacture commercial products without prior written approval from Affibody AB.

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