Antibody Internalization Assay for Fab-pHast *In-Vitro*

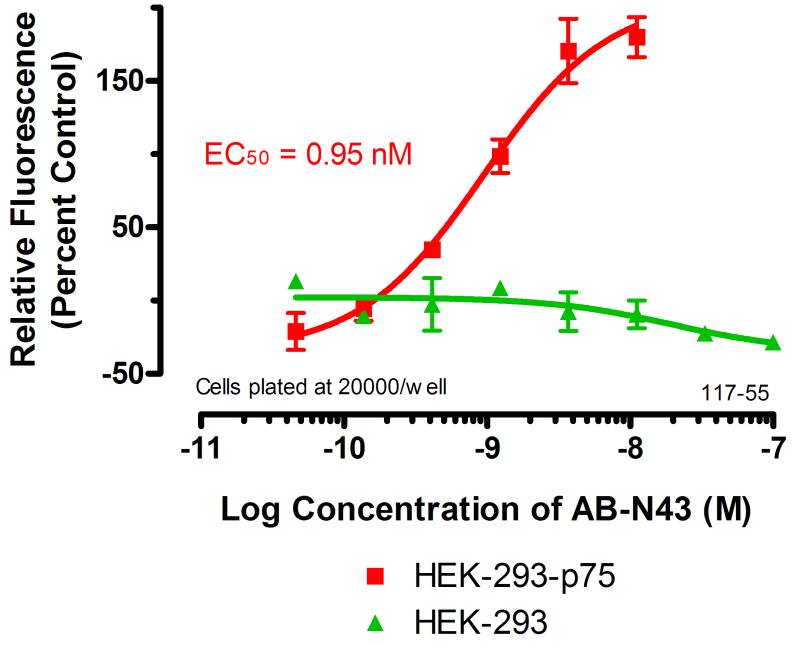
Fab-pHast human [PH-01] Fab-pHast mouse [PH-02]

100 mcg, 250 mcg, 1000 mcg

a tool to test antibody specificity, binding, and internalization with results in one (1) day

Fab-pHast in conjunction with your antibody can be analyzed via fluorescent microscope, fluorescent plate reader, or flow cytometry. In order to establish the EC50 of your antibody a fluorescent plate reader is recommended.

A sample protocol for determining internalization via Fluorescent Plate reader



pHast Ab Internalization Assay

Parental HEK-293 cells, and HEK-293 cells transfected with the p75 receptor, were plated in a 96-well plate overnight. Titrated 192-IgG antibody (**Cat. #AB-N43**) was incubated at RT with 50 nM of Fab-pHast Mouse (**Cat. #PH-02**) for 20 minutes prior to addition to cells. Plates were incubated overnight to allow maximum internalization, but a few hours is sufficient for detection.

Plates were read on a Spectra Max Gemini EM (Ex: 532nm/Em: 560nm). Data analysis was done by PRISM (GraphPad, San Diego).

Fab-pHast Internalization Protocol

1. Determine the number of cells needed for the planned number of plates. Cells are plated in the center 60 wells in 90 µl of media per well.
2. Plate cells in a 96-well black, clear bottom plate or all black plate. The clear bottom plate allows visualization of antibody internalization using a microscope. Cells are usually plated at 20,000 cells per well.
3. Transfer 100 µl of media into the wells around the edge of a 96-well plate. These wells simply offer some protection from evaporation for the experimental wells.
4. Incubate plates for 20-24 hours before treatment.
5. It's recommended to use a constant concentration of Fab-pHast at 50 nM and titrate your antibody. A good starting concentration of antibody is 10 nM. *Optimization of concentration and dilutions will need to be established. High concentrations of unconjugated antibody may act as an inhibiter of fluorescent activity.*
6. Make a stock solution of 500 nM of Fab-pHast, 10X the concentration that will be used in each well.
7. Add your desired concentrations of antibody to the Fab-pHast, 10X the desired concentration, and incubate at room temperature for 20 minutes.
8. The first and last experimental columns (2 and 11 in most plates) of cells are controls, only medium or Fab-pHast alone is added to these wells.
9. Add 10 µl of your 10X concentrations of Fab-pHast with titrated antibody to each experimental well.
10. Mix the plate gently on a plate mixer for 1-2 min, then incubate overnight to allow internalization. (Internalization can start to be detected in 1 hour, but maximal fluorescence occurs typically 19 hours after addition of the Fab-pHast conjugated antibodies).
11. To achieve a higher sensitivity, replace media with PBS before reading the plate.
12. Read the plate in a fluorescent plate reader at Ex: 532 nm/Em: 560 nm.
13. The absorbance of the sample wells is compared to the control wells to establish a curve.
14. The EC_{50} of your antibody can be derived from the curve, using PRISM software (GraphPad, San Diego).