

# A Simple Plate Based Assay Using pH Sensor Dye to Screen for Internalizing Antibody

Nidhi Nath, Becky Godat, Cesear Corona, Chad Zimprich, Mark McDougall, Poncho Meisenheimer, Marjeta Urh

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711



## 1. Introduction

Receptor mediated internalization is a key mechanism of action (MOA) for antibody drug conjugates (ADCs). However, current methods of studying antibody internalization have several limitations including:

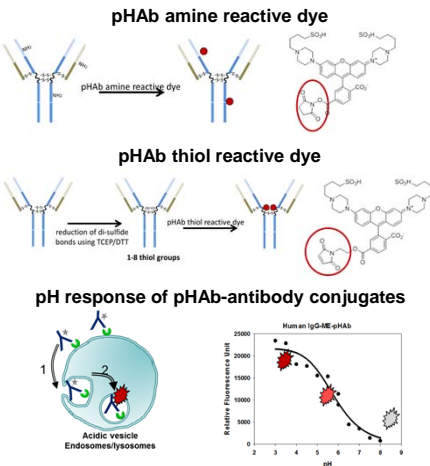
1. Multistep process not suitable for screening
2. Low signal-to-background ratios
3. Not suitable for kinetic measurements

We have developed a method that mitigates problems associated with traditional internalization assays. Method includes:

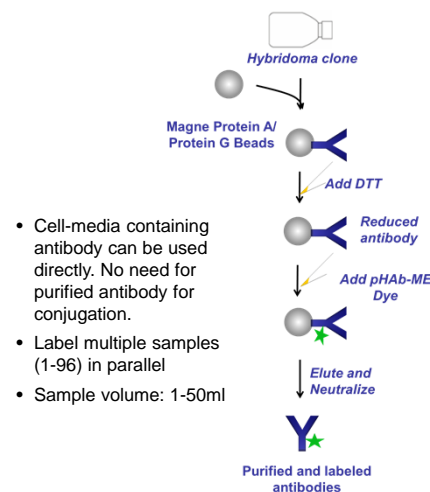
1. A water soluble, bright and photo-stable pH sensor dye (pHAb) to study internalization
2. An optimized method for conjugating pHAb dyes to antibodies directly from the cell media
3. A 96-well, plate-based assay with high signal-to-background ratios for
  - (a) real time measurement of internalization;
  - (b) screening internalization

## 2. pH Sensor Dyes (pHAb Dyes)

pHAb dyes have two different reactive groups for reaction with primary amine or thiol reactive groups.



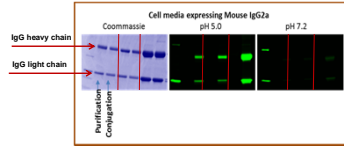
## 3. On-bead antibody-pHAb dye conjugation



Corresponding author: [nidhi.nath@promega.com](mailto:nidhi.nath@promega.com)

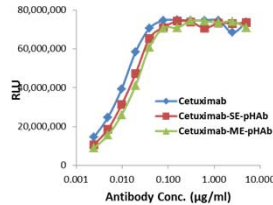
## 4. Antibody-pHAb conjugate characterization

Various samples of mouse IgG2a were conjugated with pHAb dye directly from cell media using on-bead method



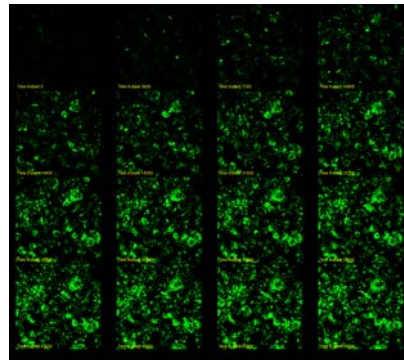
- High antibody recovery after dye conjugation
- Antibody-pHAb dye responds to pH change

Affinity of Cetuximab before and after conjugation with pHAb dye measured using ELISA



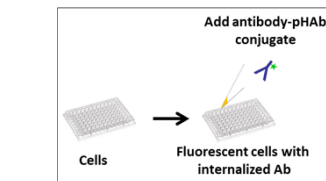
Conjugation of pHAb dye to the antibody does not change the antibody activity

## 5. Real-time internalization of Trastuzumab-pHAb conjugate



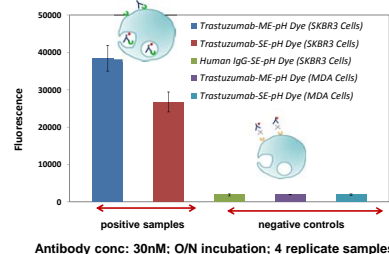
- Trastuzumab (anti-HER2) was labeled with pHAb dye
- SKBR3 cells were treated with 30nM Trastuzumab-pHAb dye
- Image captured every 60 minutes using confocal microscope to monitor internalization

## 6. Bright pHAb dyes enable 96-well, plate-based assay



### Advantages

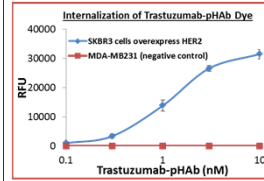
- Simple protocol: Add, replace media, read
- Excellent signal-to-background ratio



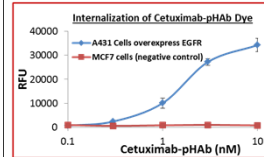
Antibody conc: 30nM; O/N incubation; 4 replicate samples

## 7. Quantitating internalization using plate-based assay

Effect of antibody concentration



A concentration series of Trastuzumab conjugated with pHAb dyes was added to the HER2 expressing SKBR3 cells or MDA-MB231 cells (negative control) in a 96 well plate. After 24hr incubation, fluorescence from the internalized antibody is measured using a fluorescence plate reader.



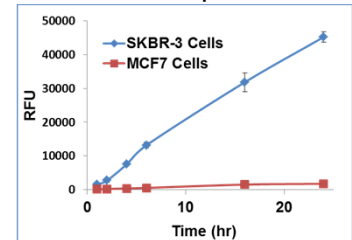
A concentration series of Cetuximab conjugated with pHAb dyes was added to the EGFR expressing A431 cells or MCF7 cells (negative control) in a 96 well plate. After 24hr incubation, we measured the signal as above.

### Advantages of plate-based quantitation:

- Easy and reproducible
- Multiple antibodies and antibody isotypes can be compared for potency
- Excellent signal to background ratio

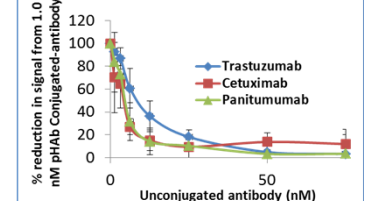
## 8. Rate of internalization and competition assay

Rate of Trastuzumab-pHAb internalization



Trastuzumab conjugated with pHAb dyes was incubated for various time with HER2 expressing SKBR3 cells or MCF7 cells (negative control) in a 96 well plate. Internalization as a function of time was read in a 96 well fluorescence plate reader.

Competition assays (1nM pHAb-IgG + unlabeled IgG)



Competition assay was done by measuring the inhibition in the internalization of pHAb conjugated antibody with unconjugated antibody.

## 9. Conclusions

This work demonstrates:

- A new, water-soluble, bright and photo-stable dye for conjugating to antibodies. Works with amine or thiol reactive groups.
- An on-bead conjugation method for labelling antibodies directly from cell media.
- A reproducible, plate-based assay that could enable antibodies to be screened and rank-ordered based on their receptor-mediated internalization properties.

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