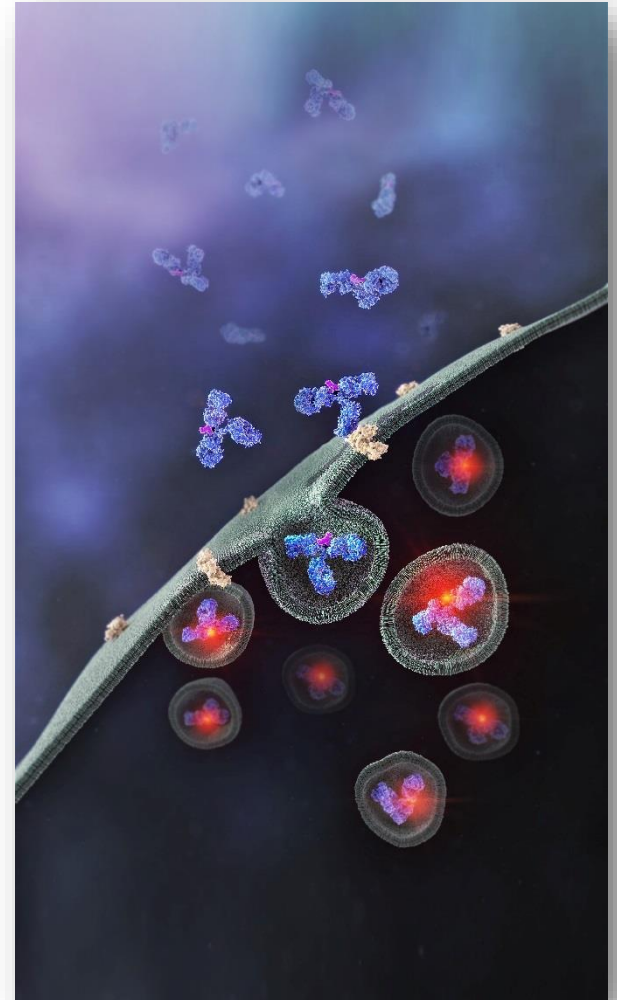


pHAb Amine and Thiol Reactive Dyes for Antibody Internalization Studies

**Nidhi Nath, Ph.D.
Group Leader, Protein Analysis
Promega Corporation**

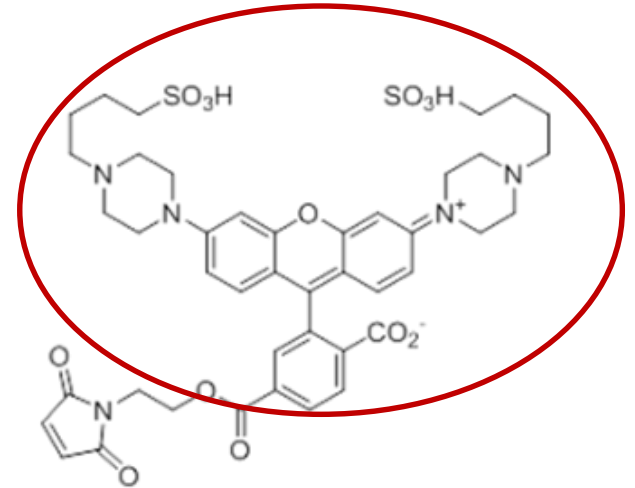
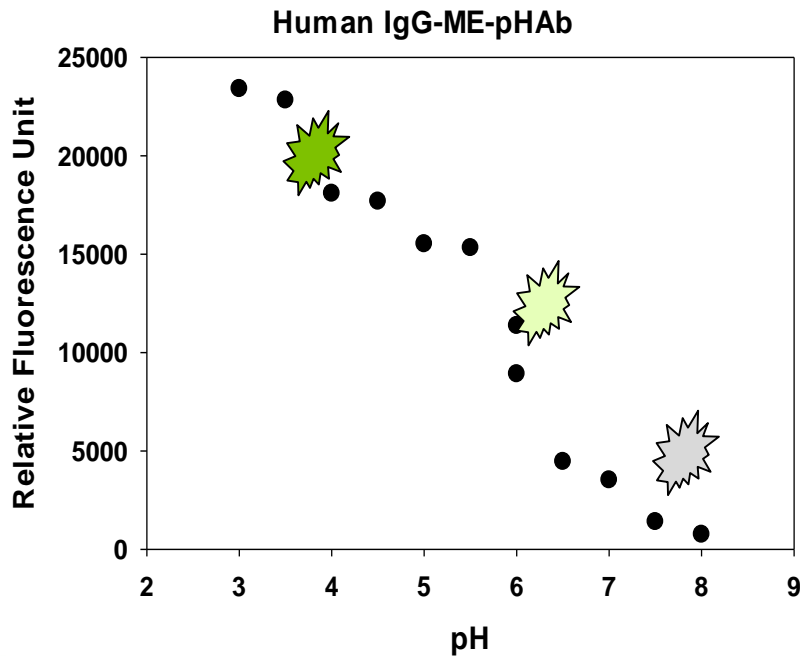
Outline

1. pHAb Dyes
2. Protocols for conjugating pHAb Dyes to antibodies
3. Applications: Internalization of pHAb conjugated antibodies



Features of pHAb Dyes

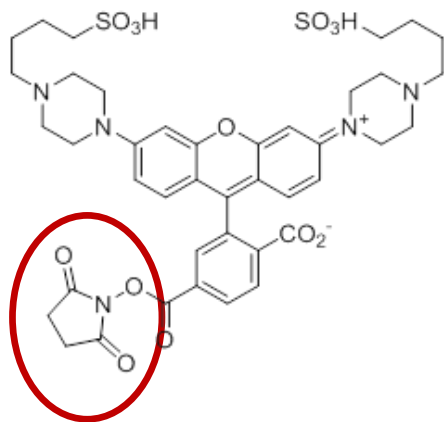
1. pHAb dyes are non fluorescent at neutral pH
2. They become highly fluorescent at acidic pH
3. pHAb dyes are sulfonated hence are hydrophilic



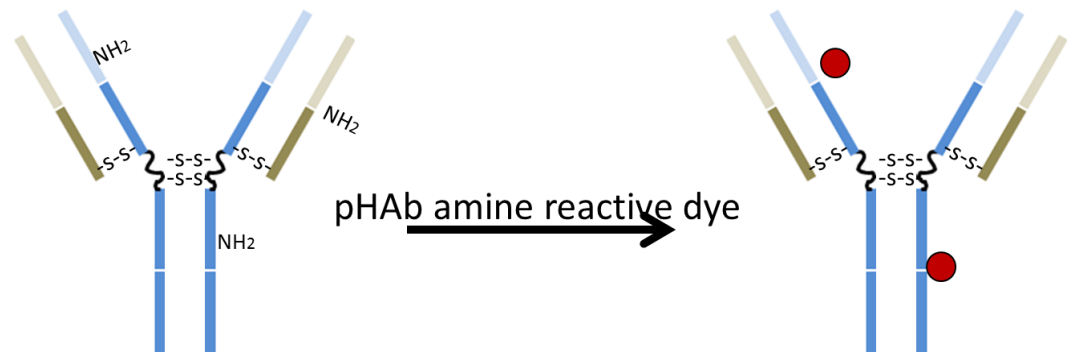
1. Excitation Wavelength: 532nm
2. Emission Wavelength: 560nm

pHAb Dyes

1) pHAb amine reactive dye



pHAb amine reactive dye

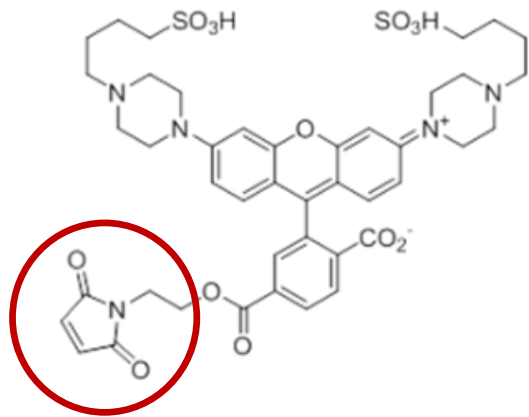


pHAb Amine reactive dye for conjugations through lysine amines

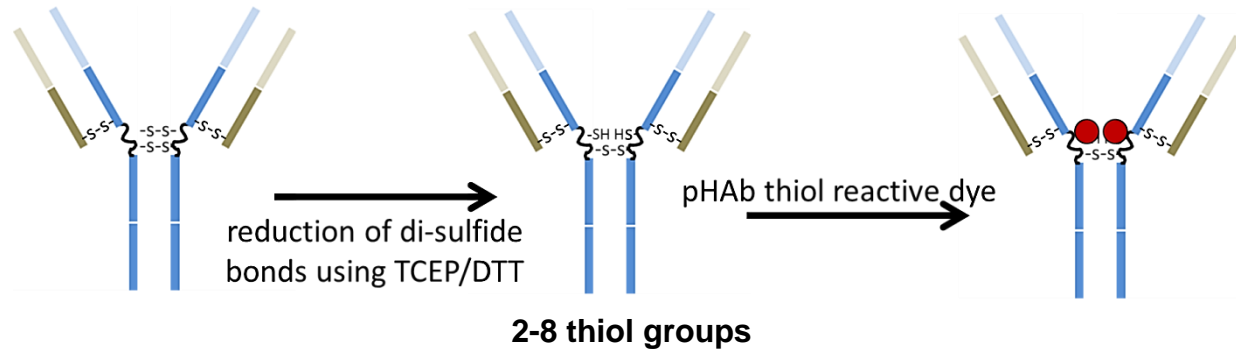
- 80-90 lysines per antibody
- ~10-40 accessible for reaction

pHAb Dyes

2) pHAb thiol reactive dye



pHAb thiol Reactive dye



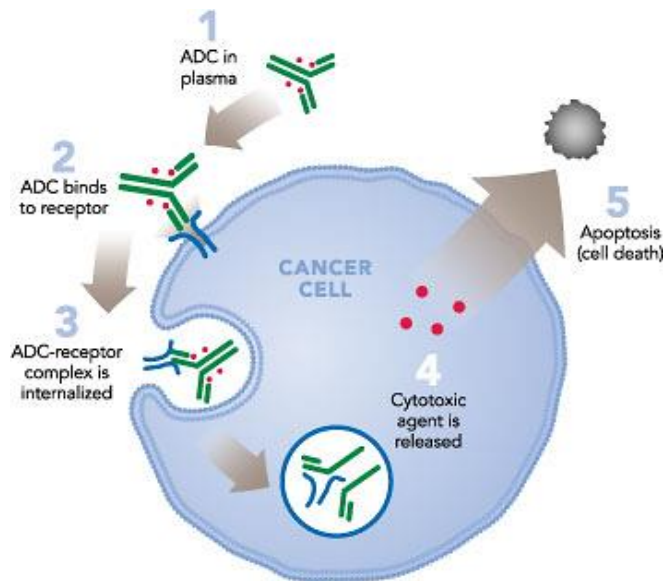
Thiol reactive dye for conjugations through thiols on reduced cysteines

- 4 di-sulfide bonds in the hinge region are available for reaction
- After reduction a total of 8 small molecules can be conjugated to the antibody

Key Applications for pHAb Dyes?

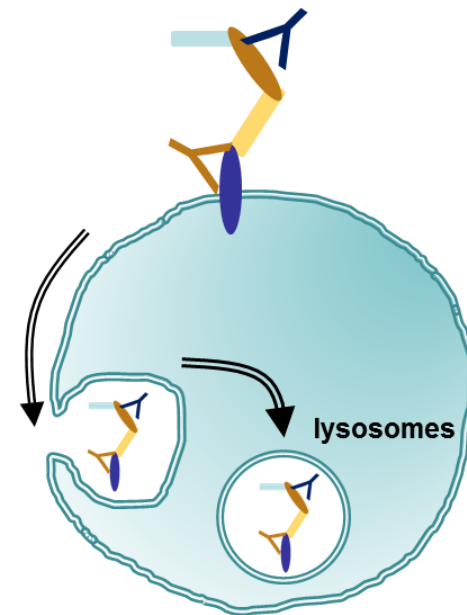
- Label antibodies and proteins to study receptor mediated antibody internalization
- Receptor mediated antibody internalization impacts (a) efficacy of antibody drug conjugates; (b) efficacy of antibody therapeutics; (c) Pharmacokinetics of antibody drug

Antibody Drug Conjugates



www.seattlegenetics.com

Antibody mixtures for therapeutic applications



Protocols for Conjugating pHAb Dyes to Antibody

1) Solution based conjugation

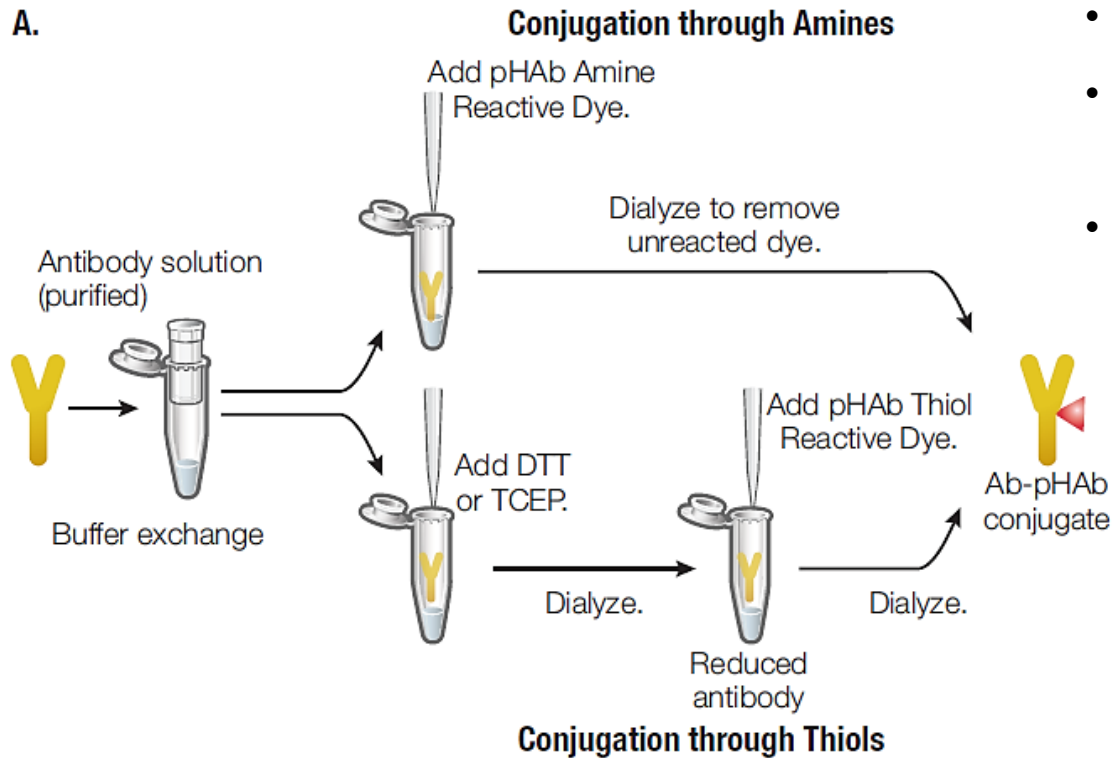
2) On-bead conjugation

Conjugation of Antibodies with pHAb Dyes

1. Three antibodies were labeled with pHAb amine- and thiol-reactive dyes
 1. Trastuzumab: Humanized IgG1
 2. Rituximab: Chimeric IgG1
 3. Human IgG: polyclonal antibody
2. Characterizing pHAb-labeled antibody
 1. Labeled antibody is visually pink in color
 2. Dye to antibody ratio: Measure absorbance at 280nm and 532nm
 3. Measure pH response of the pHAb dye conjugated to antibody
 4. Measure antigen-antibody binding affinity

Protocol: Solution Based Conjugation of Antibodies

A.

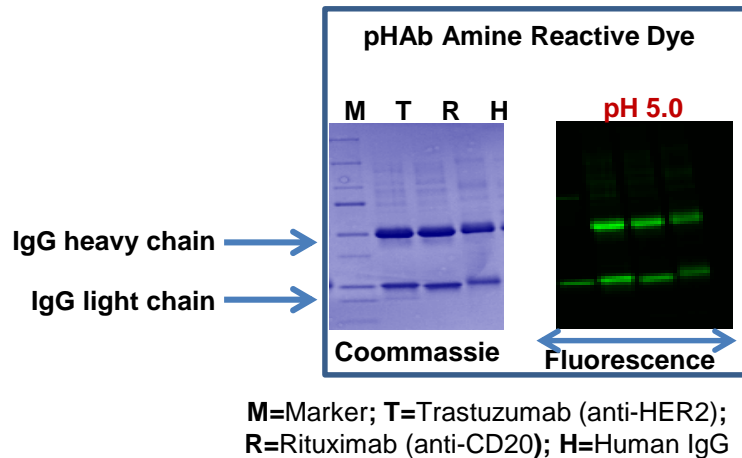


Requirements

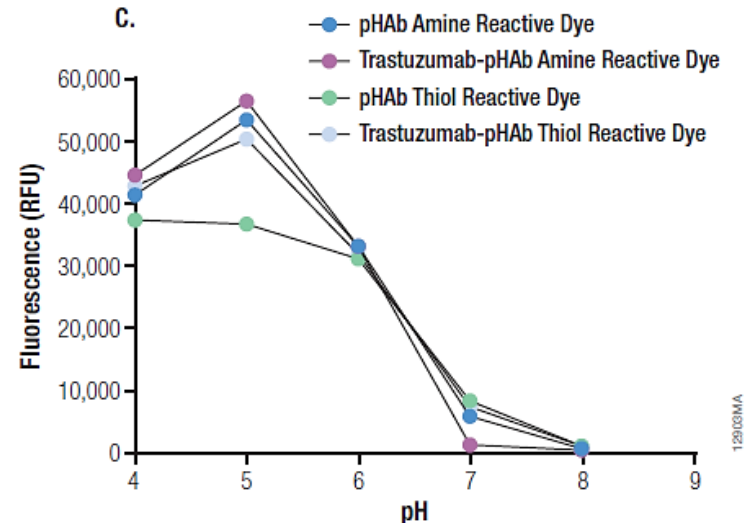
- Need purified antibody.
- Suggested starting antibody concentration: 1.0-2.0mg/ml
- Dialysis or desalting columns



Characterization of pHAb Conjugated Antibodies



pH response of the pHAb dye and pHAb dye conjugated antibody



Results

1. SDS denaturing gel shows fluorescently labeled antibody heavy and light chain
2. Typical **Dye to Antibody Ratio (DAR)** = 2-4.
Higher DAR may precipitate the antibody. Reduce the amount of added dye.
3. High speed spinning (14,000G for 1 min) after conjugation will remove any precipitated antibody if present
4. Typical antibody recoveries ~30-80%
5. Conjugated antibodies respond to pH change

Solution Based Conjugation of Antibodies

Advantages and Disadvantages

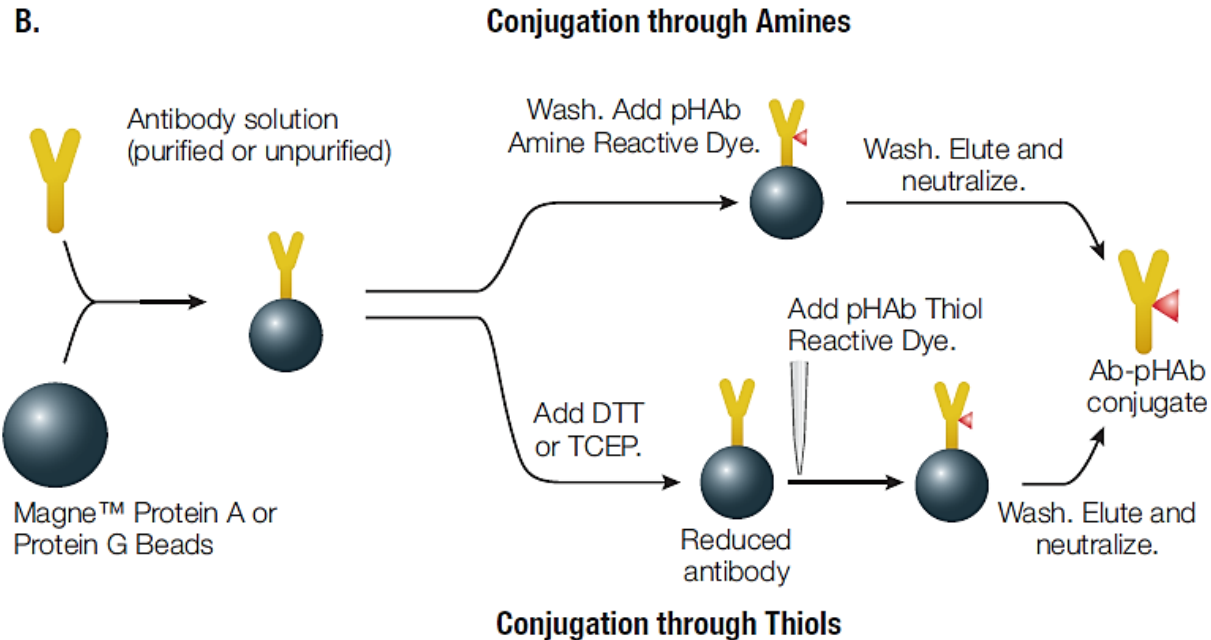
Advantages

- Number of dyes per antibody: 1-8 (2-4 is preferred)
- Any antibody/protein containing amine or thiol reactive group can be labeled with pHAb dyes
- Well established

Dis-advantages

- Need purified proteins at 1-5mg/ml
- Multiple dialysis steps leads to significant protein loss.
- Dilution of protein
- Limited throughput: Not suitable for labeling large number of proteins

On-bead Method for Antibody pHAb Dye Conjugation

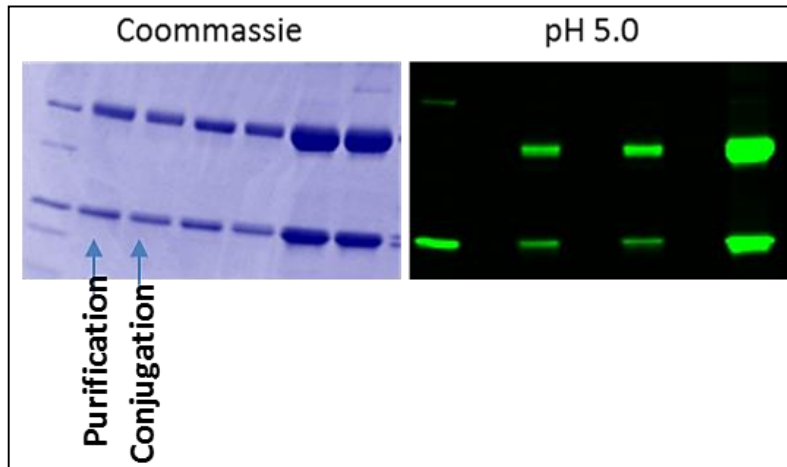


Requirements

- Purified antibody OR un-purified antibody in cell media .
- Starting antibody amount can be as low as 1.0ml of cell media containing ~100 µg/ml of antibody
- Magnetic Protein A Beads (#G8781) are preferred except for mouse IgG1 for which Magnetic Protein G beads (Cat# G7471) are preferred.

On-bead Method for Antibody pHAb Dye Conjugation

Labeling of mouse IgG2A present in cell media



Features and Advantages

- No need for purified antibody
- Combined purification and conjugation from cell-media
- Allows low amount of antibody (conc. 50 μ g/ml) to be labeled directly from cell media
- High recovery (50-80%) of antibody after conjugation
- Antibody is concentrated
- Sample from 1.0ml-50ml can be processed
- DAR: 1-3

On-Bead Conjugation

Parallel conjugation and purification at various scales

Conjugation and purification of mouse IgG isotypes in a 96-well plates

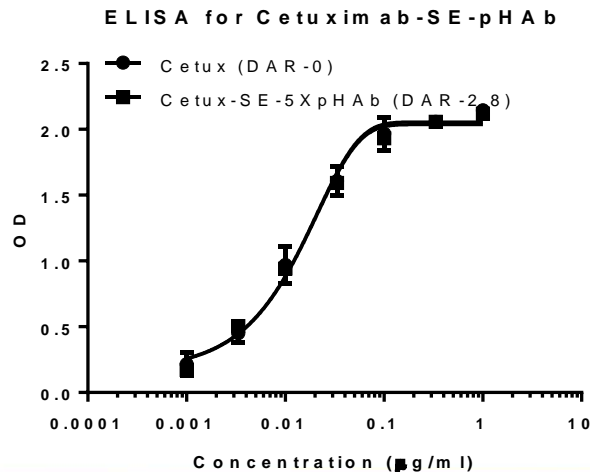
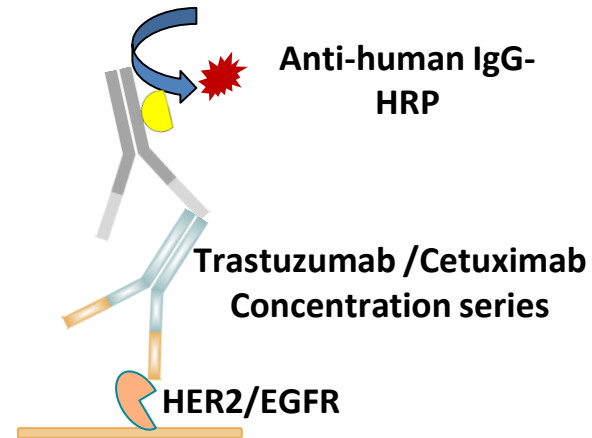
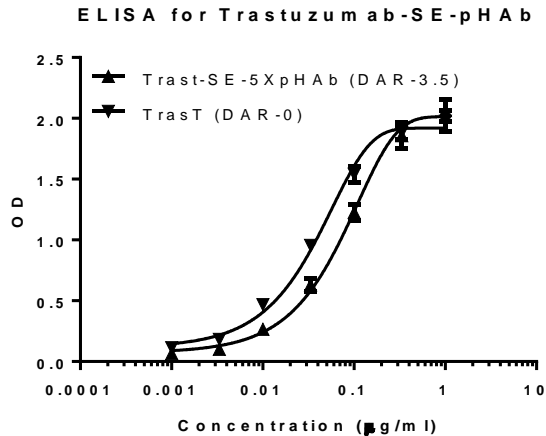
	Recovery		Dye-to-Antibody ratio
	Unconjugated Antibody (μg)	Conjugated Antibody (μg)	Conjugated Antibody
Mouse IgG1	99.5 ± 11.1	93.4 ± 3.7	0.4
Mouse IgG2a	44.4 ± 2.9	41.6 ± 0.9	0.8
Mouse IgG2b	37.0 ± 3.1	34.1 ± 1.8	1.2



96 well plate
1.0 ml sample volume

- Excellent recovery and high reproducibility

pHAb Conjugation and Impact on Antigen-antibody Binding

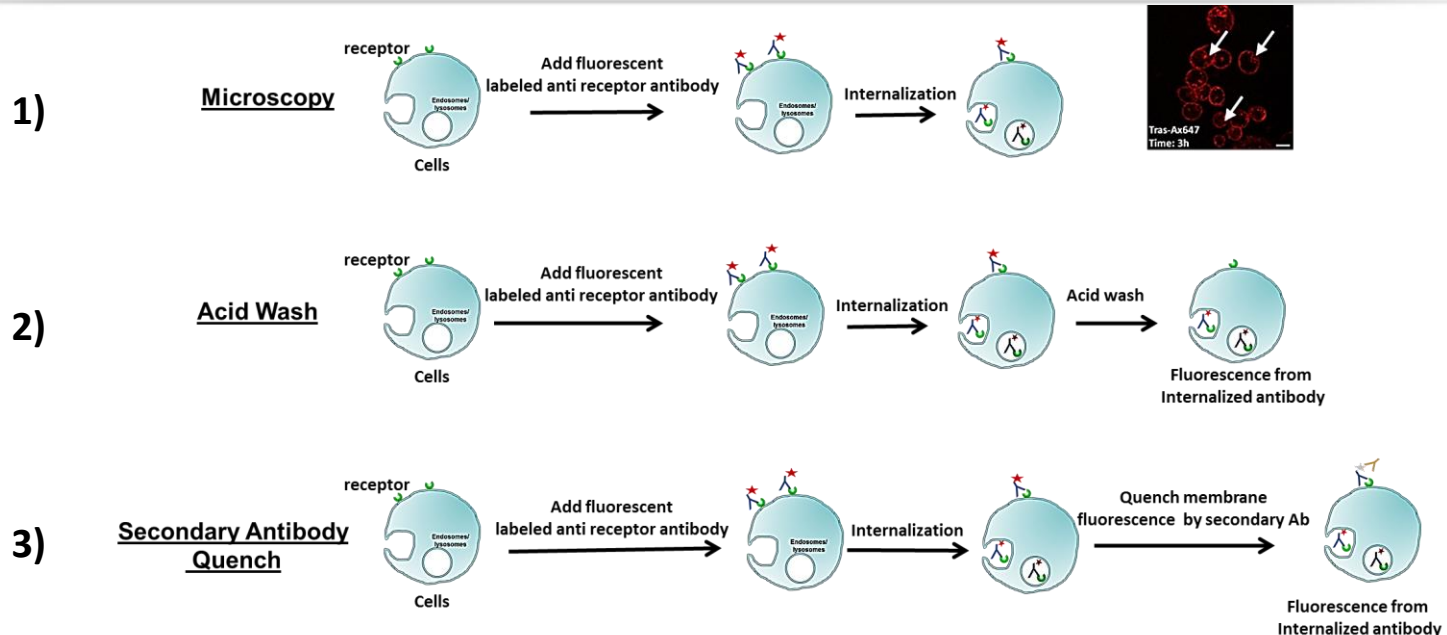


Effect of pHAb conjugation on antigen-antibody binding is dependent on:

- Antibody
- Conjugation chemistry
- Dye to antibody ratio

Internalization of pHAb Conjugated Antibodies

Current Methods to Study Internalization



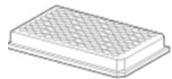
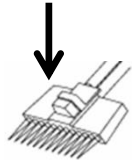
Limitations of current methods

- Needs microscopy
- Acid wash method: cell apoptosis; low signal to background due to incomplete washing
- Fluorescence quenching method: Low signal/background ratio
- Methods are low throughput and have limited kinetic measurement capabilities

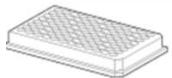
Workflow for Cell Internalization Assays Using Bright pHAb Dyes



- Cells are cultured in T75 or T150 flasks using appropriate media



- Plate cells at ~15,000 cells/well in black plate or black plate with clear bottom (for microscopy)
- Incubate for 20-24hrs



- Add pHAb conjugated antibody
- Include negative controls
 - pHAb conjugated non-specific antibodies
 - Cells with no treatment
 - Cell with non-specific or no receptor.
- Incubate (Ex. 1-24hr)

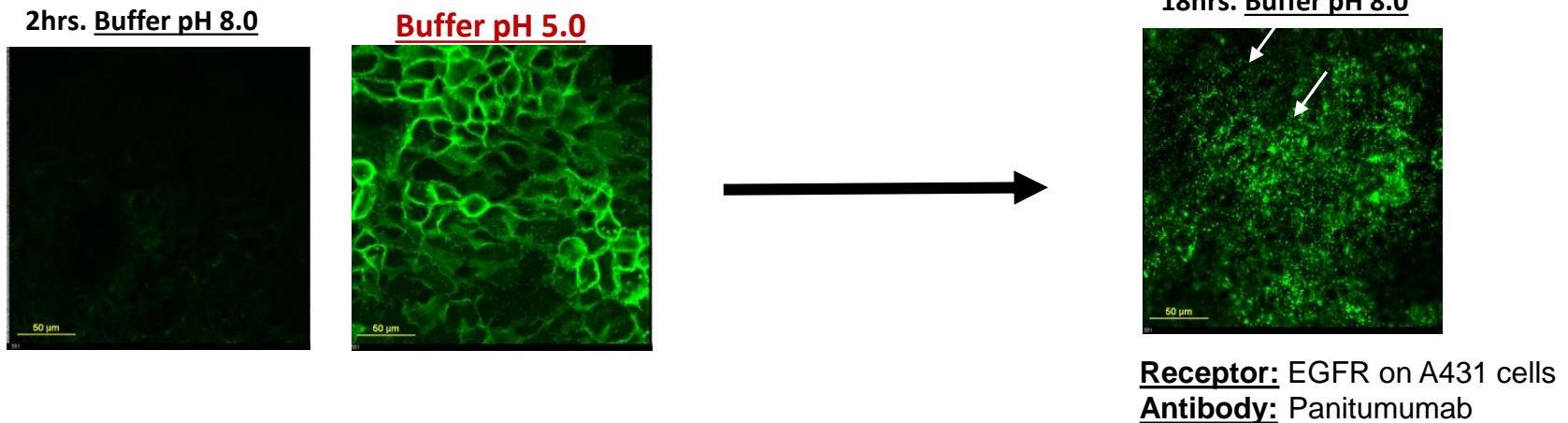


**Read plates: Microscopy,
plate reader, Flow cytometer**

Internalization of Antibody-pHAb Dye Conjugate

Simple protocol: Add and read

A431 (EGFR+) cells treated with Panitumumab-pHAb



Advantages

- No apoptosis, gentle to cell
- Excellent signal/background ratio
- Allows a plate based assay for screening purposes
- Real time internalization studies

Time Course of Internalization

- Trastuzumab (anti HER2) was labeled with pHAb dye
- SKBR3 cells were treated with 30nM Trastuzumab-pHAb Dye
- Image captured every 60 minutes using confocal
- Efficacy and potency of antibody depends on rate of internalization

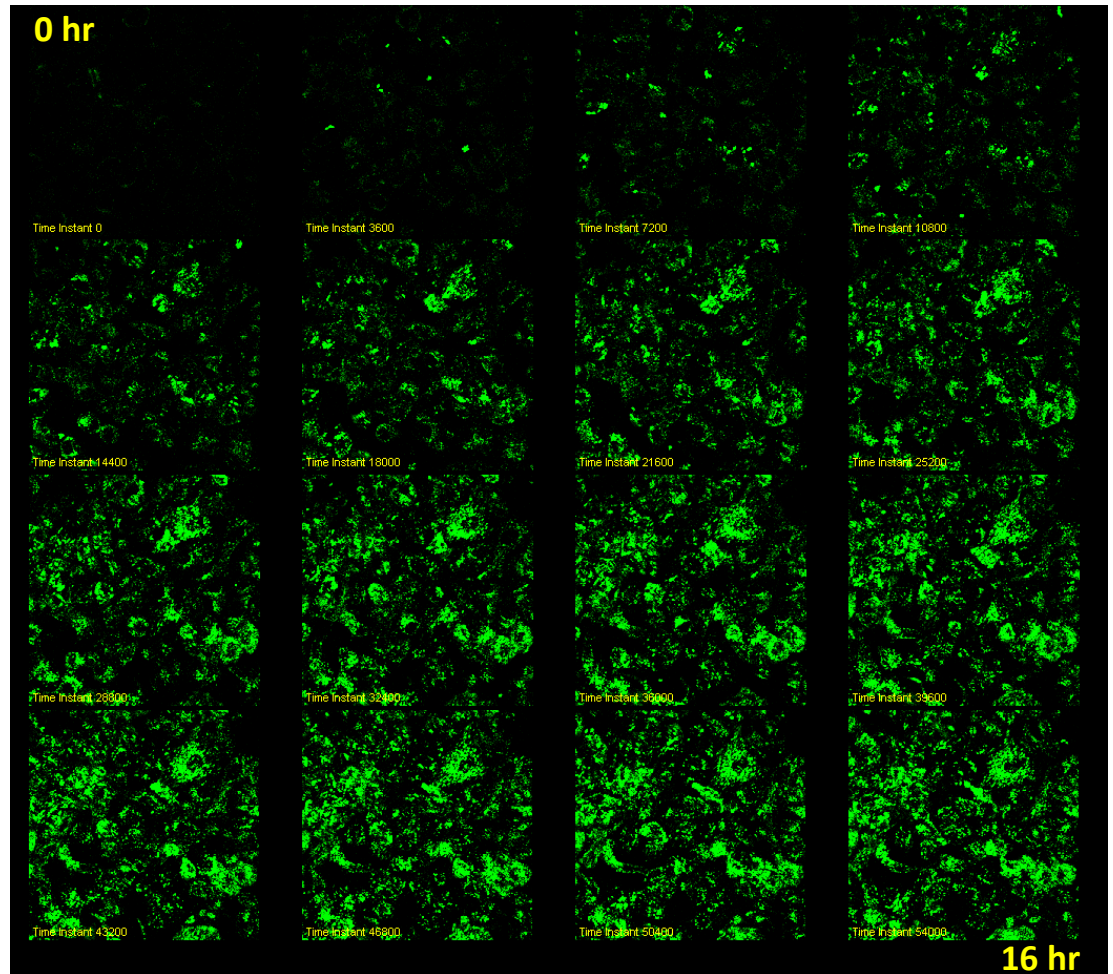
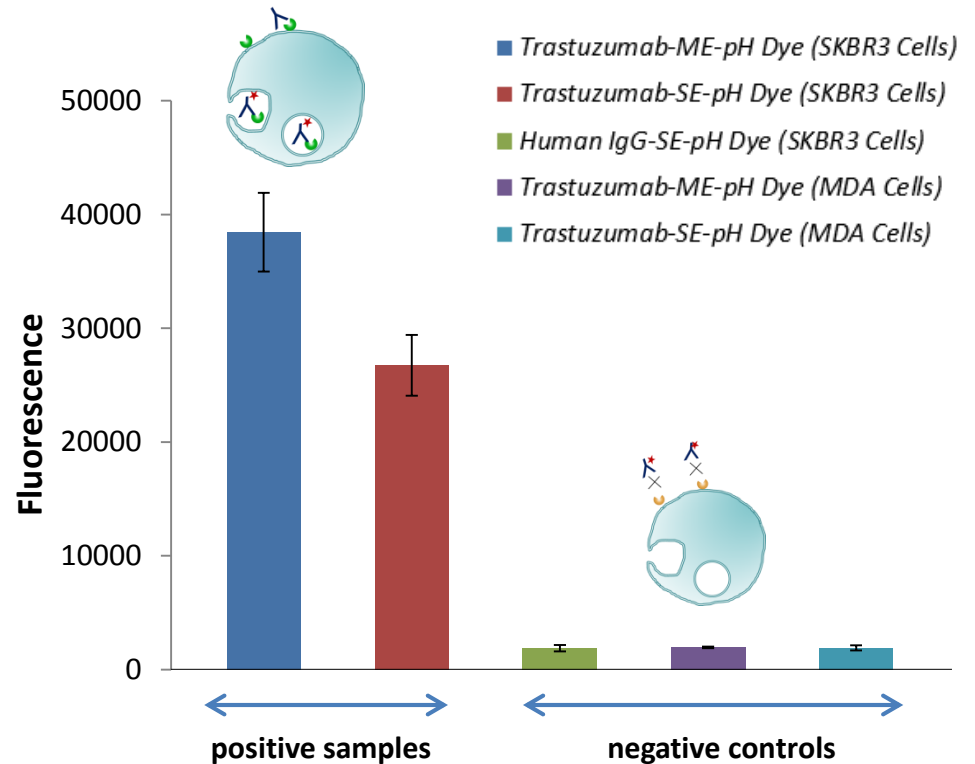
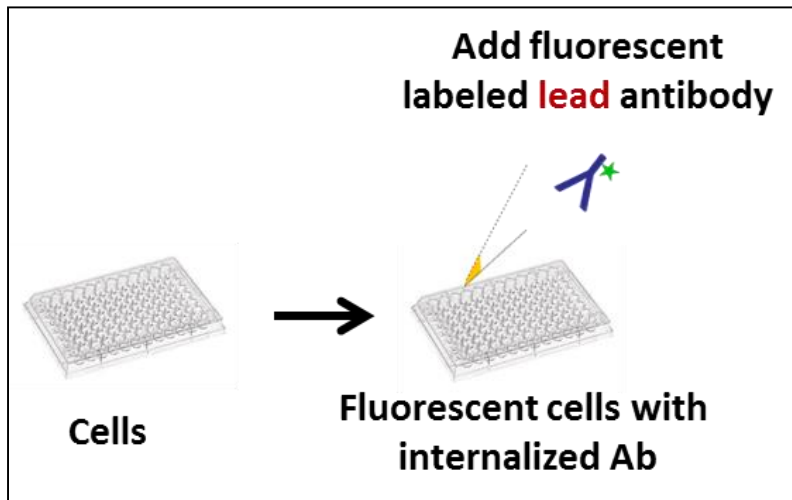


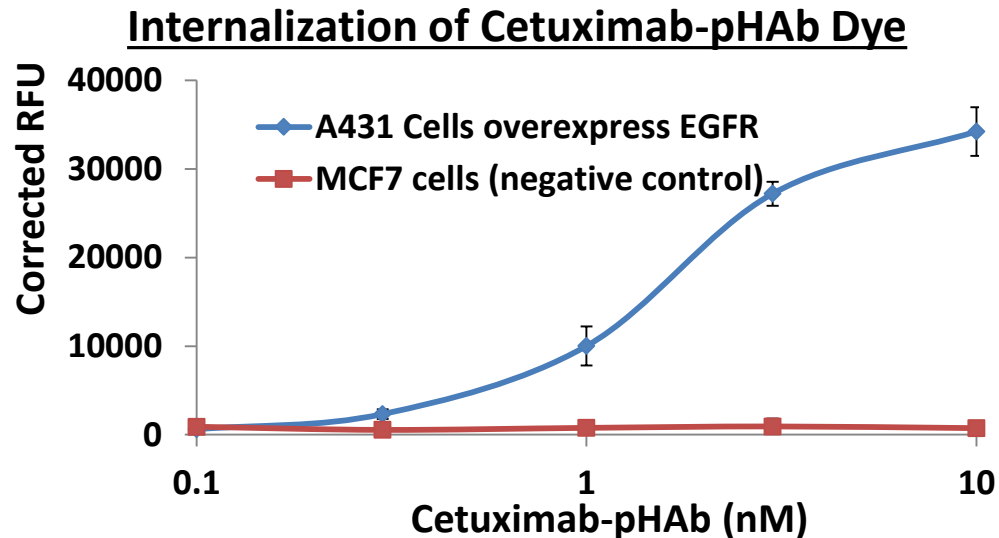
Plate Based Assays Using Antibody-pHAb Conjugates



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

- Brighter pHAb dyes will allow a plate based assay for parallel screening of large number of antibodies under various conditions.

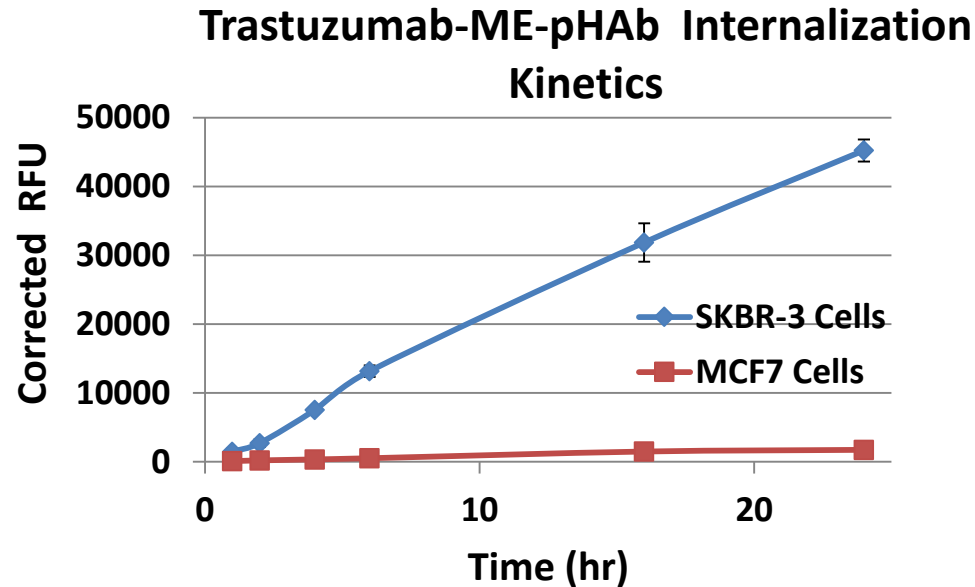
Internalization and Antibody Concentration



Advantages: Add and read protocol; Easy and reproducible;
High throughput (96 well format)

Applications: Multiple antibodies and antibody isotypes can be compared for internalization efficiency; Antibody mixtures can be screened

Kinetics of Internalization

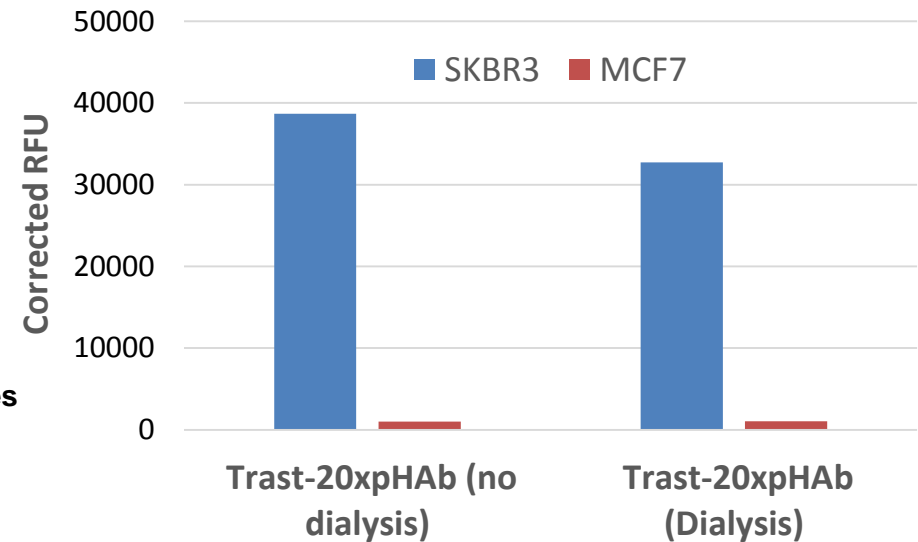
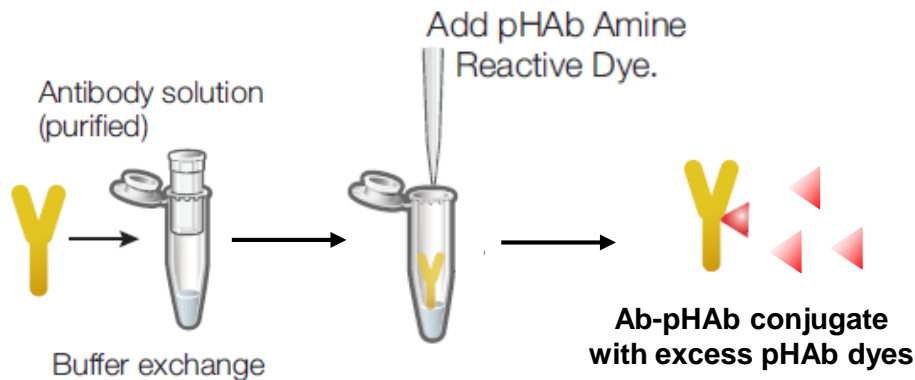


Advantages: Easy and reproducible; 96 well format

Applications: Rate of internalization impacts the efficacy of antibody drug conjugates; Internalization rate has direct correlation with receptor depletion.

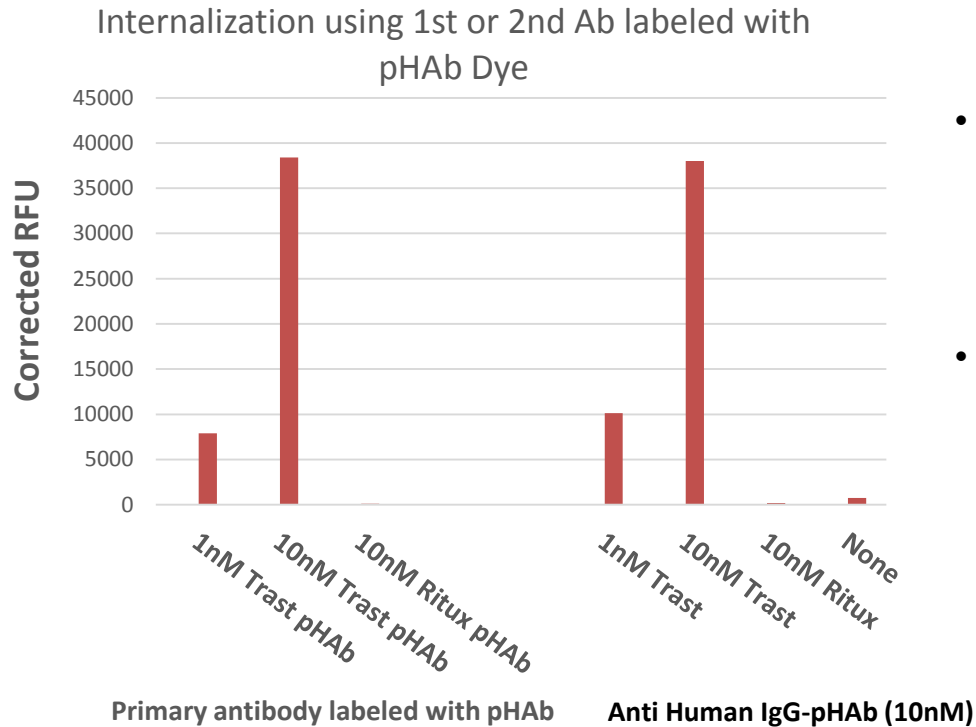
Simplified Protocol for Improving Throughput

Due to unique properties of pHAb dyes, removal of excess dye after conjugating dyes using amine chemistry is not necessary



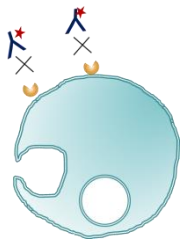
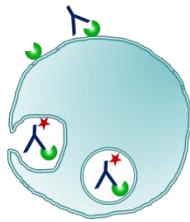
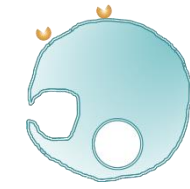
This approach will be useful where large number of antibodies with limited sample volumes are available

Using pHAb Labeled Secondary Antibodies

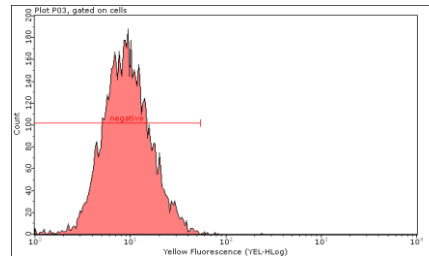


- Labeling antibodies with pHAb dyes for screening antibodies for internalization during early monoclonal development phase may not be feasible
- Secondary antibodies labeled with pHAb dyes can simplify the workflow.

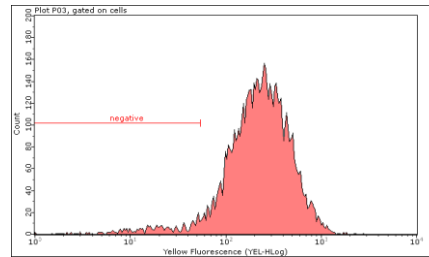
pHAb Dyes are Compatible with Flow Cytometers



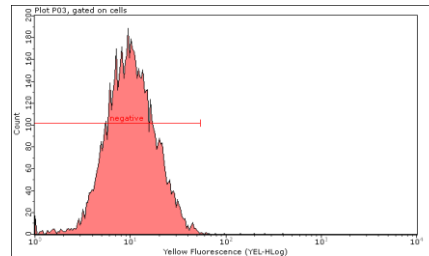
Count



Unlabeled SKBR-3 (HER2+)
99.8% negative



+30nM Trasuzumab-pHAb
5.6% negative
94.4% positive

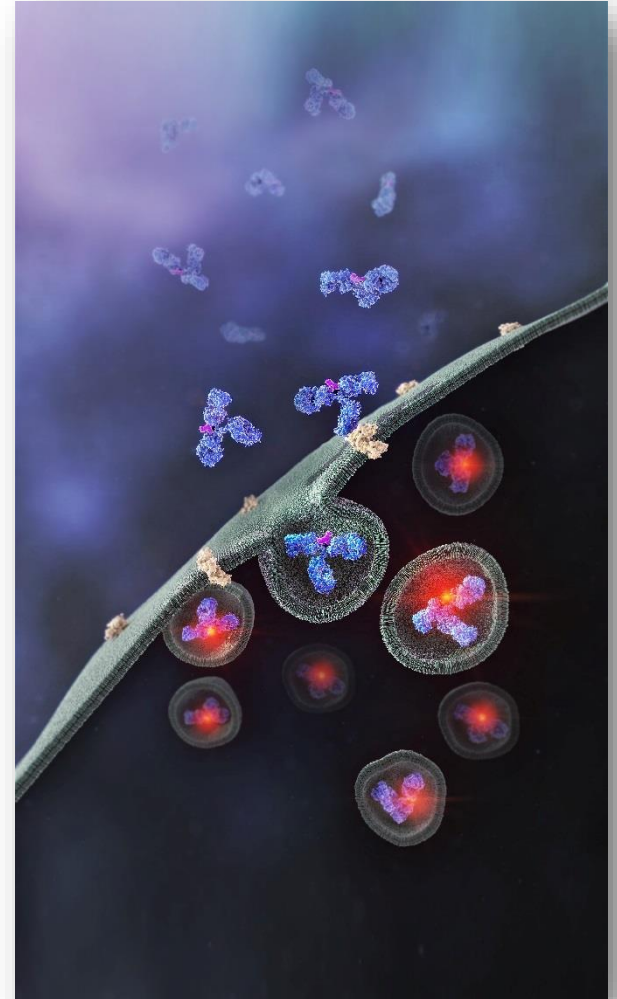


+30nM Hu IgG-pHAb
99.8% negative

Fluorescence

Summary

- pHAb dyes are pH sensor dyes that becomes highly fluorescent at acidic pH
- pHAb dyes are available in two chemistries and can be conjugated to antibodies using
 - a) reduced thiol on cysteines or
 - b) lysine residues on antibodies.
- On-bead conjugation allows combined labeling and purification of antibodies directly from the cell media in a single workflow
- pHAb conjugated antibodies enables antibody internalization studies using
 - a) Microscopy
 - b) Plate based assay
 - c) FACS



Thank you!
Questions?