TECHNICAL MANUAL

pHAb Amine and Thiol Reactive Dyes

Instructions for Use of Products G9831, G9835, G9841 and G9845

Promega



Revised 12/17 TM451

pHAb Amine and Thiol Reactive Dyes

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1. Description

pHAb Dyes

pHAb Dyes are pH sensor dyes that have very low fluorescence at pH > 7, and a dramatic increase in fluorescence as the pH of the solution becomes acidic. pHAb Dyes have excitation maxima (E_x) at 532nm and emission maxima (E_m) at 560nm. pHAb Dyes are designed specifically for labeling antibody and are available in two reactive forms suitable for antibody conjugations. pHAb Amine Reactive Dye^(a) has a succinimidyl ester group that reacts with primary amines available on the lysine amino acids on the antibodies (Figure 1, Panel A).



1. Description (continued)

pHAb Thiol Reactive Dye^(a) has a maleimide group that reacts with thiols. This maleimide group is conjugated to the antibody after the cysteine disulfide bonds in the hinge region of the antibody are reduced to thiols using a reducing agent, such as DTT or TCEP (Figure 1, Panel B).

A key feature of pHAb Dyes is that they have two sulfonate groups per dye, which improve solubility in water and reduce the aggregation often seen with other non-sulfonated dyes. pHAb Dyes maintain their fluorescence response to pH change even after conjugation to antibody, as shown in Figure 1, Panel C. Even though antibody conjugation is the key application, any protein containing primary amines on lysine amino acids or thiols on the cysteine amino acids can be conjugated with pHAb Dyes.

Conjugating pHAb Reative Dyes to Antibodies

pHAb Reactive Dyes can be conjugated to antibodies using two different workflows. First is the traditional solution-based (or in-solution) chemistry workflow (Figure 2, Panel A), which requires purified antibody at concentrations of 1.0–5.0mg/ml and several buffer exchange steps. The second workflow (Figure 2, Panel B) is on-bead conjugation, which uses magnetic protein A and protein G affinity beads to selectively capture antibodies from solutions containing purified antibody or directly from biological samples expressing antibodies (i.e., cell media). The conjugation steps are then performed on antibodies that are captured on the beads, and pHAb-conjugated antibodies are subsequently eluted using low pH buffer and quickly neutralized.

Advantages of On-Bead Conjugation:

- No need for purified antibody. Antibody purification and conjugation is achieved in a single workflow.
- Magnetic beads allow multiple antibody samples to be conjugated in parallel.
- Dilute samples containing 50µg/ml antibody can be easily conjugated with excellent recovery.
- Sample volumes from 1.0-50ml can be easily conjugated.

Applications of pHAb-Conjugated Antibodies

pHAb-conjugated antibodies can be used to monitor receptor-mediated antibody internalization. When antibody conjugated with pHAb Dye binds to its antigen on the cell membrane it exhibits minimal fluorescence. However, upon receptor-mediated internalization, antibody-pHAb conjugates traffick through the endosome and lysosomal system where pH becomes more acidic, causing the antibody-pHAb conjugates to fluoresce. Detection of this increase in fluorescence upon internalization can be accomplished using various techniques, including cell imaging, flow cytometry and fluorescent plate-based readers (1). To detect fluorescence, we recommend the use of TAMRA or Cy[®]3 filter setting on your instrument. Examples of instrument settings are:

- Fluorescence Plate Reader: Tecan Infinite[®] M1000 Pro (E_x: 532nm/E_m: 560nm)
- Flow Cytometry: Millipore Guava easyCyte 8HT using the yellow parameter (E_m filter 583/26).
- Fluorescence Microscopy: Olympus FV 500; TAMRA Setting (E_x: 543/E_m: 565)
- Fluorescence Imaging: Life Technologies EVOS-FL Cell Imaging System. RFP Red Cube (E_x : 531nm/ E_m : 593nm)
- Fluorescent Gel Scanner: GE Typhoon[®] 9410; TAMRA Setting (E_x: 532nm/E_m: 580nm)



Figure 1. Structure and pH response of pHAb Reactive Dyes. Panel A. pHAb Amine Reactive Dye (mw = 882) for labeling amines of lysine residues on the antibody. **Panel B.** pHAb Thiol Reactive Dye (mw = 907) for labeling thiols from reduced cysteines in the antibody hinge region. **Panel C.** Fluorescence is shown as a function of pH for Trastuzumab labeled with pHAb Amine Reactive Dye or pHAb Thiol Reactive Dye compared to pHAb Amine Reactive Dye or pHAb Thiol Reactive Dye alone.



1. Description (continued)



Figure 2. Schematic diagram of two workflows by which pHAb Amine and Thiol Reactive Dyes can be conjugated to antibodies. Panel A. Traditional in-solution conjugation of antibody. **Panel B.** On-bead conjugation of antibody using Magne[®] Protein A Beads or Magne[®] Protein G Beads.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
pHAb Amine Reactive Dye	1 × 250µg	G9841
	4 × 250μg	G9845
pHAb Thiol Reactive Dye	1 × 250µg	G9831
	4 × 250μg	G9835

Storage Conditions: Store the dyes at -30° C to -10° C for 1 month and below -65° C for long-term storage. **Note:** Each tube is intended for a single use.

3. pHAb Amine Reactive Dye

3.A. In-Solution Antibody Conjugation Using pHAb Amine Reactive Dye

The following tips will help you achieve the best results. The optimal buffer for labeling antibody with pHAb Amine Reactive Dye is 10mM sodium bicarbonate buffer (pH 8.5). Alternatively, use 10mM phosphate buffer (PB; pH 7.0). For successful conjugation, the antibody must be completely free of other proteins or buffers containing primary amine. Additionally, we recommend antibody concentrations of >2.0mg/ml; antibody concentrations below 2.0mg/ml may reduce conjugation efficiency.

Materials to Be Supplied by the User

(Composition of buffers and solutions is provided in Section 8.)

- amine conjugation buffer
- DMSO
- NANOpure[®] water
- desalting columns (e.g., Zeba™ columns from Pierce, Cat.# 87766 or similar) or a dialysis unit

In-Solution Antibody Conjugation Protocol

- 1. Buffer exchange the antibody in amine conjugation buffer using a desalting column.
- Quickly centrifuge the pHAb Amine Reactive Dye (i.e., 14,000 × g in a tabletop centrifuge for 5–10 seconds) and dissolve at 10mg/ml by adding 25μl of 1:1 DMSO-water mix. Mix by vortexing. It may take

1-3 minutes for the dye to dissolve completely. Make this solution just before use.

3. Add 1.2µl of pHAb Amine Reactive Dye for 100µg of antibody to make a 20 molar excess of dye.

Antibody Amount (µg)	Amount of pHAb Amine Reactive Dye (µl)
100	1.2
250	3
1,000	12



In-Solution Antibody Conjugation Protocol (continued)

- 4. Incubate for 60 minutes with mixing.
- 5. Remove the unreacted dye using desalting column. More than one column may be needed, in sequence, to completely remove the unreacted dye.
- 6. Calculate the antibody concentration and dye-to-antibody ratio as described in Section 5.

3.B. On-Bead Antibody Conjugation Using pHAb Amine Reactive Dye

The following tips will help you achieve success in amine antibody conjugation. On-bead conjugations are ideal for conjugating antibody available at low concentrations or antibodies that are not available in purified form, and thus are not suitable for traditional in-solution conjugation. Magne[®] Protein A Beads (Cat.# G8781) are recommended for on-bead conjugation except when conjugating mouse IgG1; in this case, Magne[®] Protein G Beads (Cat.# G7471) are recommended (2). Constant mixing of magnetic beads is essential for efficient conjugation. The protocol below is designed for labeling 1.0ml of sample containing 100µg/ml of antibody. We strongly recommend that you use at least 100µg of antibody for conjugation; however, smaller amounts of antibody can be conjugated with further optimization.

Recovery of pHAb Dye-conjugated antibody can vary from 50–80% depending on antibody isotype and antibody amount. Recovery of pHAb Dye-conjugated mouse IgG1 may be lower. Processing can be scaled up proportionately to 50ml using appropriately sized magnetic stands. Protocols may need to be optimized for specific antibody isotypes.

Materials to Be Supplied by the User

(Composition of buffers and solutions is provided in Section 8.)

- antibody bind buffer
- amine conjugation buffer
- elution buffer
- neutralization buffer
- DMSO
- NANOpure[®] water
- Magne® Protein A Beads (Cat.# G8781) or Magne® Protein G Beads (Cat.# G7471) for mouse IgG1
- MagneSphere® Technology Magnetic Separation Stand (two-position; Cat.# Z5332)
- tube shaker or end-over-end mixer

Equilibrate Magne® Protein A Beads

- 1. Gently shake or use an end-over-end mixer to uniformly resuspend Magne[®] Protein A Beads. Keep the suspension uniform when making aliquots of beads.
- 2. Add 50µl of bead slurry to a 1.5ml microcentrifuge tube. Place in the magnetic stand for 10 seconds.
- 3. Remove and discard the storage buffer.
- 4. Add 250µl of antibody bind buffer.
- 5. Mix and place in the magnetic stand for 10 seconds. Remove and discard the buffer.

Bind Antibody

- 6. Add 1.0ml of sample containing $100\mu g$ of antibody to the beads.
- 7. Mix sample for 60 minutes at room temperature. Keep the beads in suspension by mixing continuously.
- 8. Place tube in the magnetic stand for 10 seconds. Remove the supernatant.
- 9. Add 250µl of antibody bind buffer and mix. Place in the magnetic stand for 10 seconds. Remove and discard wash buffer.
- 10. Add 250µl of amine conjugation buffer and mix. Place in the magnetic stand for 10 seconds. Remove and discard wash buffer.

Conjugate Antibody

- 11. Add 100µl of amine conjugation buffer to the beads.
- Quickly centrifuge the pHAb Amine Reactive Dye (i.e., 14,000 × g in a tabletop centrifuge for 5–10 seconds) and dissolve at 10mg/ml by adding 25µl of 1:1 DMSO-water mix to 0.25mg of dye. Mix by vortexing. It may take 1–3 minutes for dye to dissolve completely. Make this solution just before use.
- 13. Add 1.2µl of pHAb Amine Reactive Dye for 100µg of antibody for a 20 molar excess of dye.
- 14. Mix for 60 minutes. Make sure that the beads remain in suspension with continuous mixing.
- 15. Place tube in the magnetic stand for 10 seconds. Remove and discard the supernatant.
- 16. Add 250µl of antibody bind buffer and mix. Place in the magnetic stand for 10 seconds. Remove and discard wash buffer. Repeat this step for a total of two washes.

Elute Antibody

- 17. Add 100μ l of elution buffer to the beads.
- 18. Mix for 5 minutes at room temperature.
- Place tube in the magnetic stand for 10 seconds. Remove eluted sample and transfer to a new microcentrifuge tube containing 5µl of neutralization buffer.
- 20. Quantitate the antibody concentration and dye-to-antibody ratio as described in Section 5.



4. pHAb Thiol Reactive Dye

4.A. In-Solution Antibody Conjugation Using pHAb Thiol Reactive Dye

The following tips will help you achieve success in thiol antibody conjugation. The optimum buffer for labeling antibody with pHAb Thiol Reactive Dye is 10mM phosphate buffer containing 1mM EDTA (pH 7.0). EDTA is recommended to chelate any metal ions that may re-oxidize the reduced thiols. We recommend using antibody concentrations greater than 2.0mg/ml. Lower concentrations may reduce the conjugation efficiency.

Materials to Be Supplied by the User

(Composition of buffers and solutions is provided in Section 8.)

- thiol conjugation buffer
- DMSO
- NANOpure[®] water
- 1M DTT stock solution; alternatively, 0.5M TCEP (buffered to pH 7) can be used
- desalting columns (e.g., Zeba™ columns, Pierce, Cat.# 87766 or similar) or a dialysis unit

Antibody Reduction

- 1. Buffer exchange the antibody with thiol conjugation buffer using a desalting column.
- 2. Add stock solution of DTT to a final concentration of 2.5mM.
- 3. Incubate for 1 hour with mixing.
- 4. Buffer exchange the antibody with thiol conjugation buffer using desalting column to remove DTT. **Note:** Any residual amount of DTT will interfere with the downstream application.
- Calculate the antibody concentration by measuring absorbance at 280nm. An A₂₈₀ of 1.4 corresponds to 1.0mg/ml of antibody.

Antibody-pHAb Conjugation

- 6. Quickly centrifuge the pHAb Thiol Reactive Dye (i.e., $14,000 \times g$ in a tabletop centrifuge for 5–10 seconds) and dissolve at 10mg/ml by adding 25µl of 1:1 DMSO-water mix to 0.25mg of dye. Mix by vortexing. It may take 1–3 minutes for the dye to dissolve completely. Make this solution just before use.
- 7. Add 1.2µl of pHAb Thiol Reactive Dye to 100µg of antibody for a 20 molar excess of the dye.

Antibody Amount (µg)	Amount of pHAb Thiol Reactive Dye (µl)
100	1.2
250	3
1,000	12

8. Incubate for 60 minutes with mixing.

9. Remove the unreacted dye using a Zeba[™] column. More than one column may be needed in sequence to completely remove the unreacted dye.

10. Quantitate the antibody concentration and the dye-to-antibody ratio as shown in Section 5.

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4.B. On-Bead Antibody Conjugation Using pHAb Thiol Reactive Dye

To achieve the best results please follow these recommendations: On-bead conjugations are ideal for conjugating antibodies at low concentration or that are not in purified form, and hence are unsuitable for traditional in-solution conjugation. Magne[®] Protein A Beads are recommended for on-bead conjugation except when conjugating mouse IgG1, in which case Magne[®] Protein G Beads are recommended.

Constant mixing of magnetic beads is essential for efficient conjugation. The protocol below is provided for conjugation of a 1.0ml sample containing 100μ g/ml of antibody. We strongly recommend that you use at least 100μ g of antibody for conjugation; however, smaller amounts of antibody may be conjugated with further optimization.

Recovery of pHAb conjugated antibody can vary from 50–80% depending on antibody isotype and antibody amount. Recovery of pHAb conjugated mouse IgG1 may be lower. Processing can be scaled proportionately up to 50ml using the appropriate magnetic stands. Protocols may need to be optimized for specific antibody isotypes.

Materials to Be Supplied by the User

(Composition of buffers and solutions is provided in Section 8.)

- antibody bind buffer
- thiol conjugation buffer
- 1M DTT stock solution (alternatively, 0.5M TCEP buffered to pH 7 can be used)
- elution buffer
- neutralization buffer
- DMSO
- NANOpure[®] water
- Magne[®] Protein A Beads (Cat.# G8781; or Magne[®] Protein G Beads, Cat.# G7471, for mouse IgG1)
- MagneSphere® Technology Magnetic Separation Stand (two-position; Cat.# Z5332)
- tube shaker or end-over-end mixer

Equilibrate Magne® Protein A Beads

- 1. Gently shake or use an end-over-end mixer to uniformly resuspend the Magne® Protein A Beads. Keep the suspension uniform when making aliquots of beads.
- Add 50µl of bead slurry to a 1.5ml microcentrifuge tube. Place the tube on the magnetic stand for 10 seconds.
- 3. Remove and discard the storage buffer.
- 4. Add 250µl of antibody bind buffer. Mix and place tube on the magnetic stand for 10 seconds. Remove and discard the buffer.

Bind Antibody

- 5. Add 1.0ml of sample containing 100µg of antibody to the beads.
- 6. Mix the sample for 60 minutes at room temperature. Keep the beads in suspension by mixing continuously.
- 7. Place the tube in the magnetic stand for 10 seconds. Remove the supernatant.



4.B. On-Bead Antibody Conjugation Using pHAb Thiol Reactive Dye (continued)

Reduce Antibody

- 8. Add 250µl of thiol conjugation buffer and mix. Place the tube in the magnetic stand for 10 seconds. Remove and discard the buffer. Repeat this step for a total of two washes.
- 9. Add 100µl of thiol conjugation buffer.
- 10. Add DTT to a final concentration of 2.5mM.
- 11. Mix sample for 60 minutes at room temperature. Keep the beads in suspension by mixing continuously.
- 12. Place the tube in the magnetic stand for 10 seconds and discard the buffer.
- 13. Add 250µl of thiol conjugation buffer and mix. Place the tube in the magnetic stand for 10 seconds. Remove and discard the buffer. Repeat this step for a total of two washes.
- 14. Add 100μ l of thiol conjugation buffer.

Conjugate Antibody

- 15. Quickly centrifuge the pHAb Thiol Reactive Dye (i.e., $14,000 \times g$ in a tabletop centrifuge for 5–10 seconds) and dissolve at 10 mg/ml by adding $25 \mu \text{l}$ of 1:1 DMSO-water mix to 0.25mg of dye. Mix by vortexing. It may take 1–3 minutes for the dye to dissolve completely. Make this solution just before use.
- 16. Add 1.2µl of pHAb Thiol Reactive Dye for 100µg of antibody to make a 20 molar excess of dye.
- 17. Mix for 60 minutes. Keep the beads in suspension by mixing continuously.
- 18. Place the tube in the magnetic stand for 10 seconds. Remove and discard the supernatant.
- 19. Add 250µl of thiol conjugation buffer and mix. Place in the magnetic stand for 10 seconds. Remove and discard the bind/wash buffer.
- 20. Repeat Step 19 for a total of two washes.

Elute Antibody

- 21. Add 100µl of elution buffer to the beads.
- 22. Mix for 5 minutes at room temperature.
- 23. Place the tube in the magnetic stand for 10 seconds. Remove eluted sample and transfer to a new microcentrifuge tube containing 5μ of neutralization buffer.
- 24. Quantitate the antibody concentration and dye-to-antibody ratio as described in Section 5.

5. Calculate Dye-to-Antibody Ratio

Antibody Concentration (mg/ml) = $\frac{A_{280} - (A_{532} \times 0.256)}{1.4}$

Dye-to-Antibody Ratio (DAR) = $\frac{(A_{532} \times 150,000)}{Ab \text{ Concentration (mg/ml)} \times 75,000}$

where:

Molecular weight of antibody = 150,000

Extinction coefficient of pHAb Reactive Dye = 75,000

Correction factor for pHAb Reactive Dye = 0.256

6. Measure pH Response of Antibody Conjugated to pHAb Dye (Optional)

Antibody conjugated to pHAb Dye should have an increase in fluorescence in an acidic environment. Analyze the antibody-pHAb Dye fluorescence response at pH 4 and pH 8.

- 1. Add 1µl of antibody-pHAb Dye to 100µl of 100mM citrate buffer (pH 4). Add 1µl of antibody-pHAb Dye to 100µl of 100mM phosphate buffer (pH 8). The amount of antibody-pHAb Dye may need to be increased for low concentrated samples.
- 2. Read in a fluorescence reader at $E_x 532nm/E_m 560nm$.
- 3. Calculate the fold increase in fluorescence response as follows:

Fold increase = Fluorescence of antibody-pHAb (pH 4) – Fluorescence of blank wells (pH 4) Fluorescence of antibody-pHAb (pH 8) – Fluorescence of blank wells (pH 8)

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7. Troubleshooting

Symptoms	Causes and Comments		
Antibody-pHAb Dye precipitates	High dye-to-antibody ratio may be responsible for precipitation. Reduce the amount of pHAb Dye used in the conjugation reaction.		
	Some antibodies are prone to aggregation, and a small fraction may aggregate even with low dye-to-antibody ratio. Centrifuge the sample to remove aggregates before using in downstream applications. Try adding 10% glycerol to the sample.		
	Dialyze antibody-pHAb Dye into amine conjugation buffer.		
Low recovery of antibody after Zeba™ column or dialysis	Desalting of samples may result in sample dilution as well as sample loss. The loss is dependent on antibody isotype and can be significant especially after reduction step. Try on-bead conjugation.		
Low recovery of antibody-pHAb Dye conjugate after on-bead conjugation	Efficiency of on-bead antibody conjugation will depend on the species and antibody isotype. Try in-solution conjugation.		
Conjugation to mouse IgG1	Mouse IgG1 has very low affinity for Protein A; hence, for on-bead conjugation of mouse IgG1 we strongly recommend using Magne [®] Protein G Beads (Cat.# G7471).		
Low dye-to-antibody ratio	Increase amount of dye added during conjugation.		
	Dye has lost reactivity. Inappropriate storage of dye will reduce the reactivity of the dye. Increasing the amount of dye during reaction may help.		

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8. Composition of Buffers and Solutions

amine conjugation buffer (10mM sodium bicarbonate buffer)

0.084g sodium bicarbonate

Dissolve in deionized water. Adjust to pH 8.5. Adjust the final volume to 100ml with deionized water.

thiol conjugation buffer (10mM phosphate buffer with 1mM EDTA)

0.0378g sodium phosphate, monobasic, monohydrate

0.195g sodium phosphate, dibasic, heptahydrate

Dissolve in deionized water. Add EDTA to a final concentration of 1.0mM. Adjust to pH 7. Adjust the final volume to 100ml with deionized water.

antibody bind buffer (10mM phosphate buffer)

0.0378g sodium phosphate, monobasic, monohydrate

0.195g sodium phosphate, dibasic, heptahydrate

Dissolve in deionized water. Adjust to pH 7. Adjust the final volume to 100ml with deionized water.

elution buffer (50mM glycine-HCl)

0.188g glycine

Dissolve in deionized water. Adjust pH to 2.7 with HCl. Adjust the final volume to 50ml with deionized water.

neutralization buffer (2M tris buffer)

0.472g trizma base

2.54g trizma hydrochloride

Dissolve in deionized water. Adjust to pH 7.5. Adjust the final volume to 10ml with deionized water.

9. References

- 1. Nath, N. *et al.* (2016) Homogeneous plate based antibody internalization assay using pH sensor fluorescent dye. *J. Immunol. Methods.* **431**, 11–21.
- 2. Nath, N. *et al.* (2015) On-bead antibody-small molecule conjugation using high-capacity magnetic beads. *J. Immunol. Methods.* **426**, 95–103.

10. Related Products

Product	Size	Cat.#
Magne® Protein G Beads, 20% Slurry	1ml	G7471
	5 ml (5×1 ml)	G7472
	50ml	G7473
Magne® Protein A Beads, 20% Slurry	1ml	G8781
	5 ml (5×1 ml)	G8782
	50ml	G8783
MagneSphere® Technology Magnetic Separation Stand		
(two-position)	1.5ml	Z5332
MagneSphere® Technology Magnetic Separation Stand		
(twelve-position)	1.5ml	Z5342
PolyATtract [®] System 1000 Magnetic Separation Stand	1 each	Z5410

11. Summary of Change

The following change was made to the 12/17 revision of this document:

1. References were added.

(a) Patent Pending.

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