

Determine reactivity of NHS ester biotinylation and crosslinking reagents

TR0003.1

Introduction

Many popular biotinylation and crosslinking reagents involve reactive chemistries that effect conjugation through primary amines (e.g., N-terminus and side chain of lysine residues in proteins and peptides). *N*-hydroxysuccinimide (NHS) ester is the most common amine-reactive group used in biotinylation and crosslinking reagents (see Related Pierce Products section for examples). Two forms of NHS ester reagents are available: NHS (membrane permeable, soluble in organic solvents DMF or DMSO) and Sulfo-NHS (membrane impermeable, directly water-soluble).

Although the Sulfo-NHS form is somewhat more stable, both forms of NHS esters hydrolyze rapidly in aqueous solution (within hours at pH 7; within minutes at pH 9).¹ For this reason, NHS ester reagents should be stored desiccated. To avoid condensation of moisture on the product, the bottle of reagent should be opened only after it is fully equilibrated to room temperature. Purging air from the bottle headspace with nitrogen gas before resealing it is optimal. Nevertheless, after multiple uses, i.e., repeated opening and closing of the reagent bottle, or storage under less than optimal conditions, NHS ester reagents will eventually hydrolyze, losing their ability to make the intended conjugation.

Hydrolysis as well as conjugation releases NHS as a leaving group that absorbs strongly at 260-280 nm ($\lambda_{\text{max}} = 260 \text{ nm}$; $\epsilon = 9700 \text{ M}^{-1}\text{cm}^{-1}$ in NH_4OH).² Therefore, by comparing the absorbance before and after intentional hydrolysis with strong base, one can assess the reactivity remaining in the original sample of NHS ester biotinylation or crosslinking reagent.

Important Note: Pierce biotinylation and crosslinking reagents are not quality tested by the hydrolysis method described here. Instead the reagents, which are packaged under nitrogen gas, are quality tested by other, more quantitative and accurate methods that ensure them to be of highest quality with regard to purity and reactivity. The hydrolysis method presented in this Tech Tip has not been tested with all Pierce reagents; therefore, the particular behavior (e.g., absorption properties) of each reagent in this procedure has not been established. Optimization of the method will be necessary. To establish stability data for a particular vial of reagent, newly purchased reagent can be tested by the method to establish the response of fully active reagent; then those results can be compared to the same material at later times to determine the extent of degradation.

Materials Required

- NHS ester reagent to be tested
- Dimethylsulfoxide (DMSO, Product No. 20688) or dimethylformamide (DMF, Product No. 20673), if required to solubilize NHS ester reagent; see instructions for the specific reagent.
- Phosphate (Product No. 28372) or other suitable buffer, pH 7-8. Do not use buffers that contain primary amines, such as Tris or glycine.
- 0.5-1.0 N NaOH (**Note:** Previous versions of this procedure recommended using concentrated NaOH, i.e., > 5 N. Later it was determined that concentrated NaOH degrades the NHS leaving group, eliminating its absorption at 260-280 nm).
- Spectrophotometer and quartz cuvettes capable of measuring absorbance at 260-280 nm

Procedure

1. Weigh 1-2 mg of the NHS ester reagent to be tested into a tube.
2. Dissolve reagent in 2 ml of buffer. Prepare a control tube containing 2 ml of buffer.

Note: If testing an NHS reagent that is not directly water soluble, first dissolve the reagent in 0.25 ml DMSO or DMF, and then add 2 ml of buffer. Prepare a control tube containing 0.25 ml DMSO or DMF and 2 ml buffer.

- With the spectrophotometer wavelength set to measure at 260 nm, promptly zero the instrument on the control solution and then measure the absorbance of the reagent solution. Record this absorbance value.

Note: If absorbance of the reagent solution is greater than 1.0, dilute the entire solution with additional buffer until the absorbance measures less than 1.0. Record this adjusted absorbance value.

- Add 100 µl of 0.5-1.0 N NaOH to 1 ml of the reagent solution (i.e., the same reagent solution whose absorbance value has already been adjusted to less than 1.0). Vortex tube for 30 seconds.

- Promptly measure the absorbance of the base-hydrolyzed reagent.

Note: If not measured within 1 minute, the absorbance will decrease from the initial high value obtained and lead to an inaccurate underestimate of reagent quality.

- Compare the absorbance results, using the following table:

Result	Conclusion
Absorbance of base-hydrolyzed reagent solution is measurably greater than absorbance of the starting reagent solution.	NHS reagent stock is active with respect to its amine-reactive function and can be used for bioconjugation procedures according to the product instructions.
Absorbance of base-hydrolyzed reagent solution is not measurably greater than starting reagent solution.	NHS reagent stock is hydrolyzed and inactive with respect to its amine-reactive function. Discard the reagent and purchase new product.

Related Pierce Products

- 21435** **EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit**, 25 mg of biotinylation reagent and buffers for labeling, and HABA and Avidin for labeling efficiency
- 21335** **EZ-Link™ Sulfo-NHS-LC-Biotin**, 100 mg, popular biotinylation reagent for antibodies and cell-surface labeling
- 21555** **DSS**, 1 gm, non-cleavable, amine-reactive, homobifunctional crosslinker
- 21578** **DTSSP**, 50 mg, water-soluble, cleavable homobifunctional crosslinker
- 22322** **Sulfo-SMCC**, 50 mg, heterobifunctional crosslinker ideal for making enzyme-antibody conjugates
- 20002** **Bioconjugate Techniques**, Greg T. Hermanson, Academic Press, Inc., 1995. 785 pages; softcover

Additional Information

For help in selecting an appropriate biotinylation or crosslinking reagent for your application, please visit the Pierce web site and use one of the Selection Guides. Literature references and more detailed information are available in individual product instructions, which can be downloaded from the Pierce web site or requested from Customer Service or Technical Assistance.

References

- Hermanson, G. T. (1996). *Bioconjugate Techniques*, Academic Press, Inc., p.139-140.
- Miron, T. and Wilchek, M. (1982). A spectrophotometric assay for soluble and immobilized N-hydroxysuccinimide esters. *Anal. Biochem.* **126**:433-5.

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