Instructions

Introduction:
SHNH (S-1001-010) is a bifunctional aromatic hydrazine linker used to incorporate HyNic (6-hydrazinonicotinamide) moieties onto biomolecules through their amino group via an activated ester (i.e. NHS; Figure 1). HyNic groups were developed to link Tc-99M to proteins for in vivo imaging.1-4

The number of HyNic moieties incorporated on biomolecules can be quantified calorimetrically on reaction with 2-sulfobenzaldehyde (SoluLink catalog# S-2005-100). The product yields a chromophore that absorbs at A350 with a molar extinction coefficient of 18000 (Figure 2). Procedures and calculators to guide users through this process can be found at http://www.SoluLinK.com/protocols.php

Reagents
- Desalting Spin columns (cat # S-4004-025)
- Modification Buffer (cat # S-4003-005)
- DMF (anhydrous) (cat # S-4001-005)

Equipment
- Variable-speed bench-top microcentrifuge
- Spectrophotometer or Plate Reader
- 1.5 mL microcentrifuge tubes

Note: This Protocol and all links below can be downloaded at http://www.SoluLinK.com/protocols.php

 Modification Procedure

Desalting procedure (More detailed protocols at LINK)

1. Desalt/buffer exchange the protein into Modification Buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.4).
   Notes:
   a) It is necessary to remove all free amine-containing contaminants, e.g. tris, glycine, from the protein solution before modification.
   b) High-level buffering capacity, i.e. 100 mM phosphate, is necessary for successful modification.
   c) For desalting SoluLink recommends Pierce Zeba Desalt Spin columns (# 89882) or Sartorius Vivaspin diafiltration units (#VS0112). Refer to desalting protocol for either apparatus.

A. Determine the concentration of the protein (More detailed protocols at LINK)

1. Determine the concentration of the protein to be modified using the BCA assay (ThermoScientific, #23223) or spectrophotometrically if the extinction coefficient of the protein is known.
2. Bring the concentration to 1-4 mg/mL in Modification Buffer pH 7.4

B. Prepare the SHNH Solution

1. Prepare a stock solution of SHNH in anhydrous DMF (or DMSO) by dissolving 2-4 mg of SHNH in 100 uL anhydrous DMF.
   Note:
   a) The SHNH/DMF stock solution must be used immediately.

C. Modification of protein (More detailed protocols at LINK) and of oligonucleotides (LINK)

1. Using Table 1 as a guide, add the requisite volume of SHNH/DMF to the protein solution.
   Notes:
   a) Depending on the size of the protein and the desired level of modification, the number of equivalents should be adjusted.
2. Allow reaction to incubate at room temperature for 1.5-2 hours.

![Figure 1: Scheme presenting the modification of a protein with SHNH.](image)

<table>
<thead>
<tr>
<th>IgG concentration</th>
<th>SHNH mole equivalents added</th>
<th>Determined ratio of HyNic/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>20</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>8.2</td>
</tr>
<tr>
<td>4.0</td>
<td>15</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Table 1: Recommended equivalents of SHNH linker to add to proteins at increasing concentrations to incorporate a specific linker substitution ratio.
D. Desalting procedure (detailed protocols at LINK)
1. Desalt/buffer exchange the protein into Conjugation Buffer (0.1M sodium phosphate, 0.15M sodium chloride, pH 6.0). For proteins SoluLinK recommends Pierce Zeba Desalt Spin columns (# 89882) or for oligonucleotides SoluLinK recommends Sartorius Vivaspin diafiltration units (#VS0112). Refer to desalting protocol for either apparatus.

E. Quantifying modification level (detailed protocols at LINK)
1. The molar substitution ratio (MSR) can be determined using a colorimetric reaction outlined in Figure 2. Addition of 2-sulfobenzaldehyde to a HyNic-modified biomolecule yields a bis-arylhydrazone that absorbs at 350 nm. Calculator/protocols can be downloaded from: LINK

![Figure 2: Colorimetric reaction used to quantify number of HyNic linkers on a biomolecule](image)

2. The biomolecule is now SHNH-modified and ready for conjugation to Technetium-99M or 4FB-modified biomolecules and surfaces.

Note:
   a) HyNic-modified oligonucleotides are not stable and must be conjugated or immobilized immediately following desalting.

Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein was not modified or poorly modified.</td>
<td>Protein has been contaminated with amine containing compounds</td>
<td>Desalt the protein more thoroughly with a new Zeba Spin column</td>
</tr>
<tr>
<td></td>
<td>The concentration of the protein was too low</td>
<td>Increase the concentration of the protein to &gt;2.0 mg/mL</td>
</tr>
<tr>
<td>SHNH was hydrolyzed</td>
<td>Wet or poor quality DMF/DMSO hydrolyzed the NHS ester</td>
<td>Use a good quality anhydrous DMF/DMSO to solubilize the SHNH molecule.</td>
</tr>
</tbody>
</table>

Stability
It is recommended to use the HyNic modified protein immediately. If long term storage is required it is recommended to store the modified protein <-20 °C and perform a time course stability study.

Related SoluLink Products

| S-9002-1 | S-HyNic Kit | S-4004-025 VivaSpin diafiltration device | S-2005-100 2-sulfo-benzaldehyde |
| S-1002-010 | S-HyNic | S-4001-005 DMF anhydrous | S-4023-005 Aniline |
| S-4003-005 | Modification Buffer | S-4002-005 Conjugation Buffer | S-4024-005 Aniline conjugation buffer |

References
4. For an extensive list of references see PubMed or Google Scholar: keyword HYNIC