Product Information

Recommended labeling protocols

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1. Introduction

Abberior offers a variety of fluorescent dyes with optimized properties for the labeling of biomolecules, spectroscopic studies, optical microscopy and particularly optical nanoscopy featuring superresolution. Along with non-switchable dyes (Abberior STAR series), we offer a series of caged (masked) fluorophores (Abberior CAGE series), as well as photoswitchable labels (Abberior FLIP series). Caged fluorescent dyes can be irreversibly photoactivated (uncaged) with UV- or visible light of 365-420 nm. Irradiation of the photosensitive FLIP-labels with light of 375 nm (one photon process) or 760 nm (two photon activation) results in the proton-assisted transition from the non-fluorescent spiroamide isomer to the fluorescent “open-ring” form of the same amide. Detailed descriptions of the individual features and properties of Abberior dyes (including spectra, recommended wavelengths for excitation, STED and switching, etc.) are provided on the webpage.

In general, the Abberior labels offer very good properties regarding the traditional labeling requirements, photostability, water solubility, quantum efficiency, brightness, etc. As a consequence of being well qualified for STED applications, the labels are compatible with very high depleting light intensities, which are ~100 times larger than the excitation intensity. Thus, the Abberior STAR series is particularly suitable for conventional confocal microscopy. Abberior labels cover the visible spectral range from 530 nm to 660 nm.

The useful aminoreactive reagents are N-hydroxysuccinimidy (NHS)-esters. This reactive group assists in forming a chemically stable bond between the label and the protein (antibody). For the labeling of thiol groups, the widely used reagents are maleimides, which form a stable thioether bond with the protein (antibody).

In the following are the proposed labeling protocols both for NHS and maleimide induced couplings of the label to the antibody. Additionally, Abberior offers a labeling protocol for the binding of the (secondary) antibody-coupled Abberior label with the target protein in the biological sample.

2. Labeling of antibodies/proteins with Abberior labels

The following section exemplifies the two conventional coupling techniques – NHS and maleimide induced coupling of an Abberior label with an antibody or other proteins.

Each section indicates required substances and materials. It describes the processes with the parameters to be controlled step by step.
2.1 Labeling with aminoreactive Abberior labels

Substances & Materials:

- Abberior NHS label (typically 0.2-0.4 mg)
- Antibody solution (typically 1-2 mg of protein, without BSA)
- Hydroxylamin (1.5 M in buffered solution of pH ~8.3-8.5)
- DMF or DMSO and PBS (pH ~6.5)
- NaHCO₃ (pH ~8.3-8.5)
- Gel filtration column (e.g. Sephadex G25, PD-10 with a length of ~7 cm and diameter of ~1.7 cm)
- Bradford assay

See also graphical layout of the following protocol (fig 1).

<table>
<thead>
<tr>
<th>Processes</th>
<th>Parameters to be controlled:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation of the dye solution</td>
<td>1. Dissolve dye in DMF or DMSO (10mg/ml)</td>
</tr>
<tr>
<td></td>
<td>2. Use a 15-20-fold molar excess of the dye over the antibody</td>
</tr>
<tr>
<td>Preparation of the antibody solution (1-2mg)</td>
<td>3. Add 1/10 of volume of 1 M aq. NaHCO₃ to provide pH ~8.0-8.5</td>
</tr>
<tr>
<td>Mixing of antibody with Abberior-NHS label</td>
<td>4. Add slowly the calculated amount of Abberior dye solution to the magnetically stirred solution of the antibody with aq. NaHCO₃</td>
</tr>
<tr>
<td></td>
<td>5. Gently stir at room temperature for 2 hours in the dark</td>
</tr>
<tr>
<td></td>
<td>6. Add 20 µl 1.5 M NH₂OH (pH ~8.0-8.5) to stop the reaction</td>
</tr>
</tbody>
</table>
isolation of the dye-labeled antibody

7. Equilibrate the column with 30ml PBS buffer at pH~6.5

8. Transfer the solution with the labeled antibody on top of the gel filtration column and elute it with the PBS-buffer

9. Collect fractions (0.5 ml each) obtained in the course of gel filtration chromatography

10. Determine the antibody content in each fraction, e.g. via Bradford assay

11. Determine the DOL-value (see section 2.3)

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**Fig1. Abberior label - antibody conjugation (amine-reaction)**

- **Preparing substances**
  - **Abberior NHS label**
    - Dissolve Abberior NHS label (10mg/ml) in DMF or DMSO
    - Use a 15-20 fold molar excess over the antibody

- **Antibody**
  - Add 1/10 of volume of 1 M NaHCO₃ to provide pH~8.0-8.5) to the antibody solution
  - Antibody solution must be BSA-free

- **Reaction**
  - Magnetic stirring
  - Add Abberior dye slowly to the stirred solution of the antibody
  - Incubate at room temperature for 2 hours
  - Add 20 μl 1.5 M HCl to stop the reaction

- **Separation**
  - Gel filtration (e.g. through PD-10 Sephacryl G25)
  - Perform chromatography with gel filtration column
  - Collect fractions (~0.5 ml each)
  - Determine protein content (e.g. via Bradford assay)
  - Determine DOL spectroscopically
  - For long term storage add stabilizer (e.g. BSA at a final concentration of 1-10mg/ml)

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1. Alternatively other proteins with free amine group(s)
2. Free dye (untouched by antibody)

**Storage of the protein conjugate:**

In general, the conjugate has to be stored under the same conditions as the unlabeled protein.
For storage in solution at 4°C, sodium azide (at final concentration of 2 mM) can be added as a preservative.

For long-term storage add stabilizers such as BSA (DOL needs to be determined before), separate the solution into aliquots and freeze at -20°C. Always protect from light and avoid repeated freezing and thawing.

2.2 Labeling with thiol-reactive Abberior labels (antibodies premodified with Traut’s reagent)

Substances & Materials:

- Abberior maleimide label (typically 0.2-0.4 mg)
- Antibody (with thiol groups, typically 1-2 mg of protein, without BSA)
- DMF or DMSO and PBS (pH~6.5)
- Sodium phosphate, 0.15 M NaCl, 10mM EDTA ar pH~7.2-7.5
- 2-iminothiolane (Traut’s reagent) dissolved in DMF
- Two gel filtration columns (e.g. Sephadex G25, PD-10 with a length of ~7 cm and diameter of ~1.7 cm)
- Bradford assay
- Hydroxylamin (1.5 M in buffered solution of pH~8.3-8.5)

See also graphical layout of the following protocol (fig 2).

Step 1: Modification of the antibody with Traut’s reagent

Traut’s reagent creates the requisite sulphydryls modification of the antibody necessary for conjugation with the maleimide-activated dye.

<table>
<thead>
<tr>
<th>Processes</th>
<th>Parameters to be controlled:</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Preparation of the antibody</td>
<td>1. Dissolve 1-2 mg/ml antibody in a 0.1 M phosphate buffer solution (pH= 7.2-7.6) with 0.15 M NaCl and 10 mM EDTA (1-10 mg/ml)</td>
</tr>
</tbody>
</table>
A. Traut's reagent

2. Stock solution of 2-imionothiolane in DMF (20-40-fold molar excess over antibody)

B. Mixing of antibody with Traut's reagent

3. Add solved Traut's reagent to the antibody solution (not to exceed 10% of DMF in the final solution)

4. Gently stir at room temperature for 1 hour

C. Isolation of the bioconjugate

5. Equilibrate the column with 30ml PBS buffer at pH 6.5

6. Transfer the solution with the labeled antibody on top of the gel filtration column and elute it with the PBS-buffer

7. Collect fractions (~0.5 ml each) obtained in the course of gel filtration chromatography

8. Determine antibody content (e.g., via Bradford assay) and pool fractions of containing similar concentrations of the antibody

Step II: Labeling of the Traut-modified antibody with the Abberior maleimide dye follows the protocol analogue to the NHS labeling in section 2.1.
### 2.3 Determining the degree of labeling (DOL)

The degree of labeling (DOL) – the average number of dye molecules coupled to a protein molecule (i.e., the antibody) – can be determined by absorption spectroscopy utilizing the Lambert-Beer law. The required measurement of the UV to VIS spectrum of the conjugate solution obtained after gel filtration has to be done in a quartz (UV transparent) cuvette.

The equation below represents the DOL

\[
DOL = \frac{A_{\text{max}}}{A_{\text{Prat}}} = \frac{A_{\text{max}} \cdot e_{\text{Prat}}}{(A_{280} - A_{\text{max}} \cdot C_{280}) \cdot e_{\text{max}}}
\]

while \( C_{280} \) is a correction factor of the dye given by \( C_{280} = \frac{e_{280}}{e_{\text{max}}} \).

A denotes the absorbance of the dye at a given wavelength and is defined as \( A = e \cdot c \cdot d \) where \( e \) denotes the extinction coefficient [M\(^{-1}\)·cm\(^{-1}\)], \( c \) the molar concentration [mol/L] and \( d \) the path length of the light [dm]. All absorbance values \( A \), are measured as optical density units as \([\log I/I_0]\).
The following abbreviations were used:

Representing the label properties:

\[ A_{\text{max}} \]: absorbance of the label at the absorbance maximum

\[ A_{280} \]: absorbance of the label at 280 nm

\[ \varepsilon_{\text{max}} \]: extinction coefficient of the label at the absorbance maximum

\[ \varepsilon_{280} \]: extinction coefficient of the label at 280 nm

Representing the protein (i.e. antibody) properties:

\[ A_{\text{prot}} \]: absorbance of the protein at 280 nm (absorption maximum of proteins)

\[ \varepsilon_{\text{prot}} \]: extinction coefficient of protein at 280 nm

Note: The above equation is only accurate, if the extinction coefficient of the free dye \[ \varepsilon_{\text{max}} \] at the maximum absorbance is equivalent to the value of the conjugate dye bond to the antibody (also at maximum absorbance). In practice the calculated values for the DOL can easily deviate by 20%.

3. Labeling of target proteins with antibody-coupled Abberior labels

Note that in general the mounting media and the respective protocol are highly depending on the labeling target and the application. There does not exist a general protocol of embedding, in fact usually multiple labeling options exist. You can expect, that your current protocol can be used without adaptations if you use *Abberior* labeled antibodies. However, this section gives embedding recommendations on certain applications with using Abberior labels for high-resolution microscopy.

**Substances & Materials:**

- Chemicals for
  - Fixation
  - Permeabilization
  - Blocking

  (no general recommendation possible, depends on the target of labeling)
**Fig3. Cell labeling with Abberior-antibody labels**

1. **Fixation, permeabilization and blocking**
   - Cell preparation according to standard lab protocols
   - Selection of lab protocol strongly depends on the labeling target

2. **Labeling with Abberior-antibody**
   - **Direct immunofluorescence**
     - Standard lab protocols can be performed
     - Selection of protocol dependent on target and antibody
   - **Indirect immunofluorescence**
     - Secondary antibody with Abberior label and primary antibody immunofluorescence are used according to standard lab protocols
     - Recommended is the usage of final concentration of secondary antibody with Abberior label between 1 and 10 µg/ml
     - Recommended incubation time is 1h at room temperature

3. **Embedding of the cell**
   - Embedding media are highly dependent on the application, recommendations as follows:
     - Confocal & high-resolution microscopy – Mowiol
     - Life-cell imaging – aqueous solutions, e.g. PBS or the culture medium
     - Other mounting media (e.g. glycerol,…) customized for special requirements

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- **Direct** method: Labeling of the target with a primary antibody that is labeled with an *Abberior* label

- **Indirect** method: Target is recognized by a primary antibody; a secondary antibody is labeled with the *Abberior* label and recognizes specifically the primary antibody

- Mounting media (no general recommendation possible, depends on the imaging technique)

### 3.1 *Abberior FLIP 565 – special recommendations for embedding*

We recommend to mount the *Abberior FLIP 565* labeled sample in Mowiol. This provides an environment with a high thermally induced (spontaneous) activation rate. Therefore only little UV light (approx. a few W/cm² in a widefield illumination mode) is required for sufficient switching of the compound. Illumination intensities of only a few kW/cm² are typical (for excitation at 532 nm).

Source of data: http://www.abberior.com/