B1 Labelling of capture and detection reagents

This chapter contains protocols for

- Biotinylation of capture reagent, Chapter B1.1
- Fluorophore labeling of detection reagent, Chapter B1.2
- Buffer exchange/protein concentration, Chapter B1.3

For recipes, refer to Chapter E1.

B1.1 Biotinylation of capture reagent

The following solutions and consumables are required:

- Biotinylation reagent
- Capture reagent
- Milli-Q® water
- PBS
- Protein Desalting Spin Column (Thermo Scientific), cutoff: 7K. For larger proteins consider using Nanosep 30K (Pall Life Sciences).
- 1.5-2.0 ml microcentrifuge collection tube

Step	Action
1.	**Preparation of capture reagent** Refer to supplier-provided instructions for the biotinylation reagent. The protocol in this chapter is based on instructions for EZ-Link Sulpho NHS-LC-Biotin (Thermo Scientific). Use 100 µl of 1 mg/ml capture reagent (antibody or other suitable capture reagent) for biotinylation labeling. The capture reagent to be biotinylated must be in a buffer free from stabilizing proteins (for example BSA), ammonium ions, primary amines, and sodium azide to obtain the best possible degree of labeling. • If the concentration of sodium azide in capture reagent solution is >3 mM (0.02%), remove the sodium azide, refer to Chapter B1.3. • If the capture reagent solution is in an amine-containing buffer, exchange buffer to PBS (pH 7.2 – 8.0), refer to Chapter B1.3. • If concentration of capture reagent solution is <1 mg/ml, concentrate capture reagent solution, refer to Chapter B1.3. • If concentration of capture reagent solution is >1 mg/ml, dilute with PBS. • If the capture reagent t is a powder lyophilized from an appropriate buffer, it is reconstituted in PBS.

Step	Action

2. **Preparation of Biotinylation reagent**

- Allow biotinylation reagent (EZ-Link Sulfo-NHS-LC-Biotin) to reach room temperature.
- Dissolve 0.5–1 mg of the biotinylation reagent in ice-cold deionized/distilled water (Milli-Q water) to a final concentration of 1 mg/ml.
- Vortex gently.

3. **Biotin labeling procedure**

- Mix appropriate volumes of biotinylation reagent and capture reagent solution at a 12 times molar excess of biotinylation reagent compared to capture reagent.

TIP: **Formula:**
µmol capture reagent = µg capture reagent / (g/mol) capture reagent
µmol Biotin = 12 × µmol capture reagent
µg Biotin = µmol Biotin × (g/mol) Biotin (MW Biotin = 556 g/mol)

Example:
For 100 µg of 1 mg/ml IgG (MW 150 000 g/mol), add 4.5 µg Biotin reagent
µmol IgG = 100 µg / 150 000 g/mol = 0.000667 µmol IgG
µmol Biotin = 12 × 0.000667 µmol IgG = 0.008 µmol Biotin
µg Biotin = 0.008 µmol Biotin × 556 g/mol Biotin = 4.5 µg Biotin
(4.5 µg Biotin is 4.5 µl of a 1 mg/ml Biotin reagent solution)

- Vortex gently for a few seconds.
- Incubate 1 hour at room temperature while occasionally shaking the vial gently.

4. **Removal of unbound biotinylated reagent**

- Invert the Protein Desalting Spin Column (Thermo Scientific) to suspend slurry.
- Place the column in a collection tube and centrifuge at 1500 × g for one minute to remove excess liquid.
- Add the biotinylation mixture to the column center of the compacted resin bed. Be careful not to disturb the resin or to allow sample to flow around the resin bed.
- Centrifuge at 1500 × g for two minutes. Biotinylation mixture will be collected in the tube.
- Put the tube with biotinylation mixture on ice.
- Discard the column only after a proper separation of compounds in the biotinylation mixture is confirmed.

NOTE: ***This protocol step is only valid for proteins larger than 7kD. For smaller proteins use another method for removal of free biotin, for example Nanosep (Pall Life Sciences) with a suitable cutoff.***

5. **Concentration measurement of biotinylated reagent**

- Measure absorbance (A_{280}) of the biotinylated reagent. Dilute an aliquot of the biotinylated reagent 1:10 in PBS for absorbance measurement.
- Calculate reagent concentration (mg/ml):

Reagent concentration (mg/ml) = (A_{280} × dilution factor) / ε × L

ε = 1,38 (cm^{-1} $(mg/ml)^{-1}$), L = cuvette length in cm (1 cm)

NOTE: ***The extinction coefficient, ε, for IgG is 1,38 (cm^{-1} $(mg/ml)^{-1}$), other values may be valid for other reagents.***

Step	Action
6.	**Storage of biotinylated reagent** Aliquot and store the biotinylated capture reagent at +4 °C or -20 °C (long-term storage).
7.	**Verify biotinylation** Perform a Run using 700 nM of the biotinylated molecule (for an antibody this corresponds to 0.1 mg/ml). Evaluate results to verify a successful biotinylation.

TIP: Recalculations of concentration from g/L to molar terms
Formula: M protein = g/L protein / MW (g/mol) = mol/L protein
Example: For 1 mg/mL of BSA (MW 67000 g/mol): M BSA = 1 g/L / 67000 g/mol = 0.0000149 M = 14900 nM

TIP: The concentration of biotinylated capture reagent should be high enough to saturate the streptavidin column. Normally concentrations of around 700 nM of biotinylated protein is enough to saturate the column.

B1.2 Fluorophore labeling of detection reagent

The following solutions and consumables are required

Desired Fluorophore labeling kit

- Alexa Fluor 647 Monoclonal Antibody Labeling Kit (100 µg), proteins >30 kDa
- Alexa Fluor 647 Microscale Protein Labeling Kit (20-100 µg), proteins 20-150 kDa
- DyLight 649 Microscale Antibody Labeling Kit (100 µg), 50-150 kDa
- Detection reagent

Required solutions and consumables depend on which fluorophore labeling kit is used. Please refer to supplier-provided instructions for the fluorophore labeling kit.

Step	Action
1.	**Preparation of detection reagent** Refer to instructions for the fluorophore labeling kit for details. The protocol in this chapter follows instructions given in Alexa Fluor™ 647 Monoclonal Antibody Labeling Kit (Molecular Probes) for antibodies and proteins > 30 kDa. Use 100 µl of 1 mg/ml detection reagent (antibody or other suitable detecting reagent) for fluorophore labeling. The reagent to be fluorophore labeled must be in a buffer free of stabilizing proteins (for example BSA), ammonium ions, primary amines, and sodium azide to obtain the best possible degree of labeling. • If the concentration of sodium azide in detection reagent solution is >3 mM (0.02%), remove the sodium azide, refer to Chapter B1.3. • If the detection reagent solution is in amine-containing buffer, exchange buffer to 0.1 M Sodium bicarbonate buffer (pH 7.2 - 8.0), refer to Chapter B1.3. • If concentration of detection reagent is <1 mg/ml, concentrate detection reagent solution, refer to Chapter B1.3. • If concentration of detection reagent is >1 mg/ml, dilute with 0.1 M Sodium bicarbonate buffer. • If the reagent to be labeled is a powder lyophilized from an appropriate buffer, it is reconstituted in 0.1 M Sodium bicarbonate buffer.

Step	Action

2. Fluorophore labeling procedure

- Add 1:10 volume of 1 M Sodium bicarbonate buffer to the 1 mg/ml detection reagent solution.

NOTE: ***This is not required if the starting material is lyophilized and already reconstituted in 0.1 M sodium bicarbonate buffer.***

- Transfer 100 µl of detection reagent solution to vial containing Component A (reactive dye). Vortex gently to dissolve the reactive dye.
- Protect vial from light by wrapping it with aluminum foil.
- Incubate 1 hour at room temperature while shaking the vial gently.

3. Preparation of purification column

- Moisten purification column filter with 1 ml PBS. Pressured air may be required to elute the PBS.
- Stir purification resin (Component C) and add 1 ml to the column, allow it to settle.

NOTE: ***The purification resin is a 30.000 Mw size-exclusion resin. Do not use for proteins <30 kDa.***

- Add additional purification resin until the bed volume is approximately 1.5 ml.
- Allow the column buffer to drain from the column by gravity.
- Place column in provided collection tube. Centrifuge at 1100 × g for 3 minutes.

4. Purification of fluorophore labeled detection reagent

- Load 100 µl of the labeled reagent onto the purification column.
- Place column in an empty collection tube.
- Centrifuge 1100 × g for 5 minutes.
- Transfer the labeled reagent in the collection tube to a dark vial.

5. Determination of concentration and labeling efficiency

- Measure absorbance of the purified fluorophore labeled detection reagent at A_{280} and A_{650}. Dilute an aliquot of the fluorophore reagent 1:10 in PBS for absorbance measurement.
- Calculate detection reagent concentration:
  Concentration (M) = $(A_{280} - (A_{650} \times 0.03)) \times$ dilution factor / ε,
  $\varepsilon = 203000\ (cm^{-1}\ M^{-1})$

NOTE: ***The extinction coefficient, ε, for IgG at A_{280} = 203000 ($cm^{-1}\ M^{-1}$), other values may be valid for other reagents.***

- Calculate degree of labeling (moles fluorophore per mole of detection reagent):
  Labeling degree = (A_{650} × dilution factor) / ε × Concentration (M))
  $\varepsilon = 239000\ (cm^{-1}\ M^{-1})$ of fluorophore at A_{650}

NOTE: ***For antibodies a degree of labeling of 3–7 moles of fluorophore dye per mole of antibody is recommended.***

6. Dilution and storage of fluorophore labeled detection reagent

- Dilute the fluorophore labeled reagents to 1000 nM in PBS, 0.2% BSA (Calbiochem).
- Aliquot and store fluorophore labelled detection reagent at –20 °C protected from light.

Step	Action
7.	**Verify fluorophore labeling** • For all new assays, assay formats and newly labelled reagents, perform a titration Run to find the optimal concentration for the detection reagent, refer to Chapter A3.4.2. • For an established assay, perform a test Run using previously established concentration. Compare the result from the new batch of labelled reagent with results from previous batches in previous titration Runs.

B1.3 Buffer exchange, azide removal and protein concentration protocols

This chapter contains protocols for buffer exchange and reagent concentration.

B1.3.1 Nanosep 30K Protocol

The Nanosep 30K protocol (Pall Life Sciences), is used for buffer exchange and azide removal. Nanosep columns come in different cut off values (3, 10, 30 and 100K). The optimal filter size of the spin column depends on the size of the reagent.

The following solutions and consumables are required

- Nanosep 30K membrane (Pall Life Sciences) and sample reservoir
- Deionized water
- 1×PBS without NaN_3

Step	Action
1.	• Pre-rinse the Nanosep by filtering 500 µl deionized water or buffer through the membrane twice. • Spin up to 11 000 × g for a couple of minutes. Do not allow the membrane to dry out prior to use.
2.	• Pipette the required volume of protein (50 to 500 µl) into the sample reservoir. • Fill up to 400 µl with buffer. • Cap the Nanosep device. • Spin up to 11 000 × g for 2 – 20 minutes.
3.	• Wash 2–3 times with 200 – 400 µl buffer. • Spin up to 11 000 × g for 2 – 20 minutes.
4.	If the reagent appears to have spun dry in the device, the reagent is easily recovered by pipetting several times with approximately 50 µl buffer.
5.	Dilute the reagent with buffer to get a concentration of 1 mg/ml.

B1.3.2 Thermo Scientific spin column protocol

The protocol is copied from Thermo Scientific Protein Desalting Spin Columns, product number: 89849, which is designed for use on proteins with a M_W > 7000 Da.

Follow this protocol when using Desalting spin column for buffer exchange and azide removal.

The following solutions and material are required

- Thermo Scientific Protein Desalting Spin Column
- Variable-speed bench-top microcentrifuge
- 1.5–2 ml microcentrifuge collection tubes
- Equilibration buffer: PBS

A Protein Desalting Spin Columns Preparation

Step	Action
1.	Prepare the protein desalting spin column • Invert column to suspend slurry. • Twist off bottom closure and loosed cap. • Place column in 1.5–2 ml microcentrifuge collection tube. • Centrifuge at 1500 × g for 1 minute to remove excess liquid.
2.	Perform four column washes • Add 400 µl of equilibration buffer to the top of column. • Centrifuge the column at 1500 × g for 1 minute to remove excess liquid. • Discard buffer from the collection tube.

Sample Loading

Step	Action
1.	• Place column in a fresh collection tube and remove the cap. • Apply 30–120 µl of sample (proteins with M_W greater than 7000) to the center of the compacted resin bed. Be careful not to disturb the resin or to allow sample to flow around the resin bed.
2.	**Optional:** To improve recovery percentage of low molecular weight proteins or for small sample volumes, add 20–40 µl of equilibration buffer to top of resin. Do not exceed a total of volume of 120 µl.
3.	Centrifuge column at 1500 × g for 2 minutes. The desalted sample is collected in the collection tube.
4.	Discard the desalting column after use.

B1.3.3 Concentrate reagent solution

This protocol for reagent concentration uses Nanosep 30K (Pall Life Sciences). Nanosep columns come in different cut off values (3, 10, 30 and 100K). The optimal filter size of the spin column depends on the size of the reagent.

The following solutions and consumables are required

- Nanosep 30K membrane (Pall Life Sciences) and sample reservoir
- Deionized water
- 1×PBS without NaN_3.
- Thermo Scientific spin column protocol

Step	Action
1.	• Pre-rinse the Nanosep by filtering 500 µl deionized water or buffer through the membrane twice. • Spin up to 11 000 × g for a couple of minutes. Do not allow the membrane to dry out prior to use.
2.	• Pipette the required volume of protein (50 to 500 µl) into the sample reservoir. • Cap the Nanosep device. • Spin up to 11 000 × g for 2 – 20 minutes.
3.	If the reagent appears to have spun dry in the device, the reagent is easily recovered by pipetting several times with approximately 50 µl buffer.
4.	Dilute the reagent with buffer to get a concentration of 1 mg/ml.