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# ab272214

## Biotin conjugation kit (with inbuilt quantification)

A product of Expedeon, an  
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Biotin conjugation kit (with inbuilt quantification):

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For Conjugation of Antibodies to Biotin.

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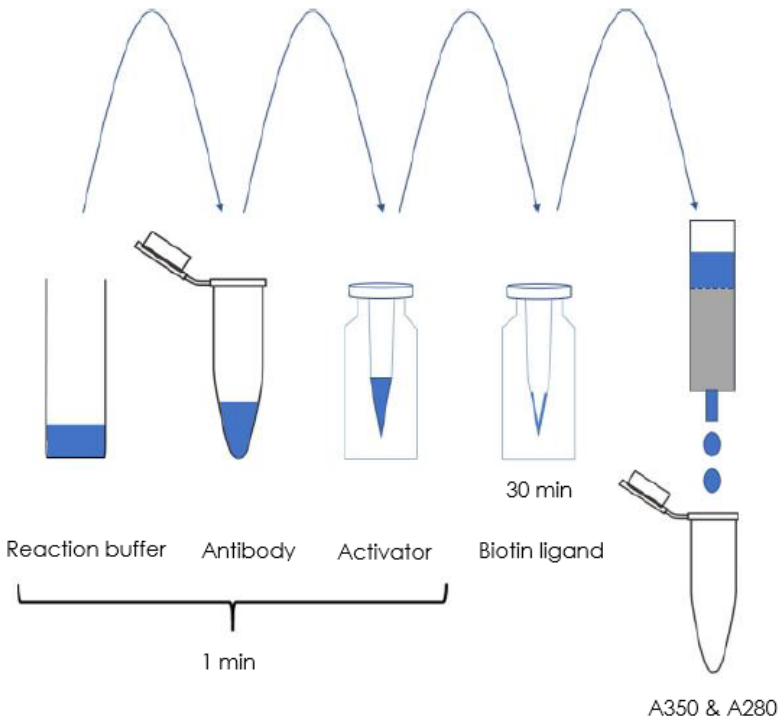
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# 1. Overview

Biotin conjugation kit (with inbuilt quantification) (ab272214) is a unique antibody/protein conjugation system with inbuilt biotin quantification, which obviates the need for a separate determination of incorporated biotin using the HABA assay.

The conjugation procedure is extremely simple: the antibody to be labeled is used to reconstitute a freeze-dried activator compound, and the resulting mixture is added to the biotin chromophore ligand vial. After a fixed time, the conjugate is desalted to remove excess ligand and the absorbance of the conjugate is read at  $A_{350}$  and  $A_{280}$ . These absorbance values allow you to determine the yield of conjugate, its concentration, and the average number of biotins per antibody.



## 2. Materials Supplied and Storage

Remove the Columns and Column equilibration buffer and store at room temperature. Store the remaining components at -20°C in the dark immediately on receipt. Kit can be stored for 1 year from receipt, if components have not been reconstituted.

Avoid repeated freeze-thaws of reagents.

Item	3 x 100 µg	1 x 1 mg	Storage temperature
NAP-5 Column	3 unit	-	RT
PD-10 Column	-	1 unit	RT
Column equilibration buffer	1 unit	1 unit	RT
Biotin ligand	3 vials	1 vial	-20°C
Activator	3 vials	1 vial	-20°C
Quencher reagent	3 vials	1 vial	-20°C
Reaction Buffer	1 vial	1 vial	-20°C
DMSO	1 vial	1 vial	-20°C

### 3. Technical considerations

#### 3.1 Pre-Conjugation Considerations:

Buffer components	
pH	6.5-8.5
Amine free buffer (e.g. MES, MOPS, HEPES, PBS)	Yes
Non-buffering salts (e.g. sodium chloride)	Yes
Chelating agents (e.g. EDTA)	Yes
Sugars	Yes
Glycerol	Not tested
Thiomersal / Thimerosal	No
Merthiolate	No
Sodium Azide	<0.1%
BSA	No
Gelatin	No
Tris	No
Glycine	No
Proclin	No
Borate buffer	Not tested
Nucleophilic components (Primary amines e.g. amino acids or ethanolamine and thiols e.g. mercaptoethanol or DTT)	No

**Δ Note:** Compounds above marked 'not tested' yet are expected to be tolerated but no confirmatory data is available at this time.

### 3.2 Recommended amount and volume of antibody for optimal results:

Vial Size	Recommended Amount of Antibody ( $\mu\text{g}$ )	Fixed Volume of Antibody ( $\mu\text{L}$ )
100 $\mu\text{g}$	100	100
1 mg	1000	1000

***Δ Note:** The recommended conjugation conditions are based on using a 1 mg/mL antibody concentration. Antibodies greater than 1 mg/mL should be diluted to 1 mg/mL using amine-free buffer.*

***Δ Note:** Antibodies less than 1 mg/mL ideally should be concentrated, but if this is impractical you may still be able to label the antibody. While there is no definite lower limit of acceptable concentration, in general we would not label antibodies at <0.5 mg/mL. The volume of antibody used in the reaction must always be the recommended one, thus you cannot add more volume to compensate for a low antibody concentration.*

### 3.3 Controlling biotinylation efficiency

As not all antibodies will label with equal efficiency it may be necessary to optimize the conditions for any new antibody. The easiest way to control the number of biotins per antibody is to vary the reaction time. Under standard conditions (30 minute reaction), you can expect about four biotins per antibody. If you require a lower number of biotins, the reaction time can be reduced to 15 minutes, or even 10 minutes. Very short reaction times should be avoided as the effect of any small timing errors will be magnified.

It should also be noted that it is impossible to obtain one biotin on every antibody molecule. A population of conjugates always exists, and the calculated biotin density is the population average. To ensure that virtually all antibodies will have at least one biotin tag, you will need to aim for higher

average biotin density, e.g. 2. The optimum level of biotinylation for each application must be determined empirically, but the easy quantification of the biotin ligand means that the characteristics of a 'good' batch can be reliably determined, and similar batches of material can be prepared in the future. The HABA assay by comparison is very inconsistent and thus it is much harder to characterize and replicate conjugates.

## 4. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.

**Δ Note:** *The melting point of DMSO is 19°C and some warming in the hand may be needed.*

### 4.1 Conjugation reaction

- 4.1.1 For each biotinylation reaction you will first need to set up and equilibrate one NAP-5 or PD-10 column. Remove the top cap from the column(s) and pour off the excess liquid. Next, remove the bottom cap and secure the column(s) in a rack or clamp. You will need to have sufficient space below the column(s) to position a tray to collect waste liquid and, later, a rack with Eppendorf tubes to collect the biotin conjugates.
- 4.1.2 Completely fill the column with column equilibration buffer. Allow this volume to pass into the column bed and collect the waste into a tray. Repeat this operation four more times. The column is then equilibrated and ready for use. There is no need to reattach the end caps to the column.
- 4.1.3 Prepare the biotin ligand. Add dry DMSO to the biotin vial (volume provided in table below). (The ligand is present in a thin film at the bottom of the tube and may not be easy to see). Re-cap the vial and hold the tube firmly by the cap. Flick the bottom of the tube a few times to dissolve the ligand. Leave the tube of dissolved ligand on the bench for later use and start to prepare the antibody.

Vial Size	Volume dry DMSO
100 µg	10 µL
1 mg	100 µL

- 4.1.4 To the volume of the 1 mg/mL antibody solution specified in step 3.2 add the reaction buffer and mix by gently pipetting up and down.

Vial size	Antibody volume	Volume reaction buffer
100 µg	100 µL	10 µL
1 mg	1 mL	100 µL

- 4.1.5 Reconstitute a vial of freeze dried Activator with antibody mixture. The Activator should dissolve instantly.

Vial Size	Antibody mixture
100 µg	110 µL
1 mg	1.1 mL

- 4.1.6 Immediately transfer the antibody/Activator mixture to the vial of DMSO-reconstituted biotin ligand from step 4.1.3. Eject the mixture quickly to encourage rapid mixing of the DMSO solution and continue pipetting up and down for 2-3 seconds.

- 4.1.7 Leave the vial for 30 minutes at room temperature. (See Section 3.3 for guidance on controlling the extent of biotinylation).

- 4.1.8 At the end of the reaction time, add the quencher and proceed to steps 4.1.9 & 4.1.10 below as quickly as possible.

Vial Size	Quencher
100 µg	10 µL
1 mg	100 µL

- 4.1.9 For the 100 µg vial size, add 100 µL of column equilibration buffer.

- 4.1.10 Transfer the contents of the vials to the column(s) prepared in step 4.1.2. You will be transferring:

Vial Size	Conjugate
100 µg	~220 µL
1 mg	~1.2 mL

- 4.1.11 Allow the samples to pass completely into the column bed (at which point the column will stop dripping) before moving to the next step.
- 4.1.12 Position a tube marked 'FT' (flow through) directly below the column outlet. Add equilibration buffer to the column and collect the eluted material. The conjugate should not be present in the flow through but keep this tube as a precaution. The tube can be discarded later once the conjugate has been recovered.

Vial Size	Volume of equilibration buffer
100 µg	400 µL
1 mg	1.8 mL

- 4.1.13 Position a clean collection tube marked 'C' directly below the column outlet and add equilibration buffer. This volume will push the biotin conjugate out of the column. Any free biotin ligand is retained within the column bed. Do not throw any tubes or columns away until you have confirmed in step 4.1.14/4.1.15 that the biotin conjugate is in collection tube C ('sample C').

Vial Size	Volume of equilibration buffer
100 µg	500 µL
1 mg	2 mL

- 4.1.14 Take absorbance readings at two wavelengths:  $A_{350}$  and  $A_{280}$ . For accurate measurements it is strongly recommended that you use a standard quartz cuvette with a 1 cm path length.
- 4.1.15 Before taking measurements at each wavelength, blank the spectrophotometer with column equilibration buffer. Read sample C. An  $A_{280}$  value of  $>0.2$  should be observed, which

reflects the amount of antibody and the incorporated biotin ligand. The  $A_{350}$  value (a measure of ligand alone) depends on the reactivity of the antibody with ligand and the reaction time (normally 30 minutes). Since the biotin ligand absorbs at both wavelengths, a correction factor is applied to the measured  $A_{280}$  value to ensure that the amount of antibody calculated from the  $A_{280}$  value is correct (Section 5).

## 4.2 Conjugate Storage

Storage at 4°C is recommended for any conjugate. A preservative may be desirable for long-term storage. A preservative may be desirable for long-term storage up to 2 years. Other storage conditions (e.g. frozen at -70°C or stored at -20°C with 50% glycerol) may also be satisfactory. The best conditions for any new conjugate must be determined by experimentation.

## 5. Calculating Number of Biotins per Antibody and Recovery of Conjugate

The concentration of biotin is determined directly from the  $A_{350}$  measurement. The value is multiplied by  $10^6/19,474$  to give the  $\mu\text{M}$  concentration of biotin (A). The antibody conjugate concentration is obtained from a corrected  $A_{280}$  value, after subtracting the product of  $A_{350} \times 0.4475$  from the measured  $A_{280}$  value. The corrected  $A_{280}$  is then multiplied by  $10^6/210,000$  to give the  $\mu\text{M}$  concentration of antibody (B). The number of biotins per Ab is obtained by dividing A by B.

Finally, the % recovery is obtained by multiplying the concentration of antibody by:

Vial Size	Multiplier
100 $\mu\text{g}$	74.96
1 mg	29.85

These multipliers and correction factors assume that the kit protocol has been followed exactly. A more detailed description of how these factors are derived is given in the next section, which is important if you cannot follow the recommended protocol or you wish to label biomolecules other than antibodies.

### 5.1 Detailed explanation of calculations

5.1.1 This section need only be read if you are interested in understanding the basis of the calculations. The calculations rely on the standard equation for absorbance  $A = ecl$ , where  $e$  = extinction coefficient,  $c$  = concentration and  $l$  = path length. Since a standard cuvette has a path length of 1 cm, the equation simplifies to  $A = ec$ . It follows that concentration =  $A/e$ .

5.1.2 To understand the units of  $e$  we rearrange  $A = ecl$  to  $e = A/cl$ . Absorbance is dimensionless, thus the units of  $e$  are  $\text{M}^{-1}\text{cm}^{-1}$ . The extinction coefficient for biotin ligand =  $19,474 \text{ M}^{-1}\text{cm}^{-1}$ . An  $A_{350}$  absorbance reading of 0.15 (i.e. measure of the

biotin chromophore) would mean that the concentration (A/e) is 0.15/e (M). It is usually more convenient to express the equation as  $c = 10^6 \times A/e$  to give a concentration expressed in  $\mu\text{M}$ .

- 5.1.3 With antibody biotin conjugates, the concentration is determined in a similar fashion (except of course that the extinction coefficient for antibodies, 210,000, is used). The measured  $A_{280}$  value is inflated because of the biotin chromophore, but as the amount of biotin ligand is precisely known (from the  $A_{350}$  value) its contribution to  $A_{280}$  value can be calculated as  $A_{350} \times 0.4475$ . Thus, we subtract this result from the measured  $A_{280}$  value. The corrected  $A_{280}$  value is then used to determine the concentration of antibody conjugate: Corrected  $A_{280} \times 10^6/210,000$  gives the  $\mu\text{M}$  concentration of the Ab-biotin conjugate.
- 5.1.4 The recommended antibody concentration in the kit protocol is 1 mg/mL (6.67  $\mu\text{M}$ ), but the final concentration of conjugate is lower because of dilution in the column separation procedure. This increase in volume must be considered when the overall recovery of antibody conjugate is determined.

$$\% \text{ Recovery} = 100 \times \frac{\text{Final volume } (\mu\text{L}) \times \text{Conc. Ab conjugate } (\mu\text{M})}{\text{Original volume } (\mu\text{L}) \times \text{Conc. Antibody } (\mu\text{M})}$$

- 5.1.5 For the 100  $\mu\text{g}$  vial size, the final volume of conjugate is 500  $\mu\text{L}$ , the original volume of antibody is 100  $\mu\text{L}$ , and the original concentration is 6.67  $\mu\text{M}$ . By consolidating these constants in the numerator (i.e. 100 x 500  $\mu\text{L}$ ) and denominator (i.e. 100  $\mu\text{L}$  x 6.67  $\mu\text{M}$ ) we arrive at a single correction factor of  $50,000/667 = 74.96$ .
- 5.1.6 For the 1 mg vial size the final volume of conjugate is 2000  $\mu\text{L}$ , the original volume of antibody is 1000  $\mu\text{L}$ , and the original concentration is 6.67  $\mu\text{M}$ . By consolidating these constants in the numerator (i.e. 100 x 2000  $\mu\text{L}$ ) and denominator (i.e. 1000  $\mu\text{L}$  x 6.67  $\mu\text{M}$ ) we arrive at a single correction factor of  $20,000/667 = 29.99$ .

- 5.1.7 The calculated  $\mu\text{M}$  concentration of the antibody-biotin conjugate is simply multiplied by the relevant correction factor from 4.4.5 or 4.4.6 to give % recovery.
- 5.1.8 In case you cannot follow the recommended protocol, the full equations are summarized below, which can be substituted with the appropriate numbers if your volumes vary or if proteins with different extinction coefficients are to be biotinylated:

(i) Concentration of biotin ligand =  $10^6 \times A_{350} / [e \times l]$  ( $\mu\text{M}$ ) ( $l$  is normally 1)

(ii) Contribution of the dye to  $A_{280}$  value =  $A_{350} \times 0.4475$

(iii) Concentration of conjugate =  $10^6 \times (A_{280} \text{ value} - [A_{350} \text{ value} \times 0.4475]) / [e \times l]$  ( $\mu\text{M}$ )

(iv) Recovery =  $100 \times [\text{Vol. protein at end } (\mu\text{L}) \times \text{concentration } (\mu\text{M})] / [\text{Vol. protein at start } (\mu\text{L}) \times \text{concentration } (\mu\text{M})]$

***Δ Note:*** The  $10^6$  terms above apply because extinction coefficients [ $e = A/c \times l$ ] are given in units of  $\text{M}^{-1} \text{cm}^{-1}$ . If you use a protein other than an antibody check that its extinction coefficient is expressed in units of  $\text{M}^{-1} \text{cm}^{-1}$ . If not, you will need to adjust the equations accordingly.

## 5.2 Worked example

Goat anti-rabbit IgG (100  $\mu\text{L}$  of 1mg/ml; 6.67 $\mu\text{M}$ ) was labeled with biotin for 30 min and 500  $\mu\text{L}$  of conjugate was recovered from the NAP-5 column. The following absorbance data were obtained:

$A_{350}$ : 0.086

$A_{280}$ : 0.246

Other relevant parameters:

Extinction coefficient biotin ligand = 19,474  $\text{M}^{-1} \text{cm}^{-1}$

Extinction coefficient of antibody = 210,000  $\text{M}^{-1} \text{cm}^{-1}$

Step 1 is to calculate biotin concentration =  $10^6 \times 0.086 / 19,474$  ( $\mu\text{M}$ )

Thus, biotin concentration = 4.416  $\mu\text{M}$

Step 2 is to determine how much of the signal at  $A_{280}$  is caused by the biotin ligand rather than by the antibody.

$$\text{Ligand contribution to } A_{280} = A_{350} \times 0.4475 = 0.086 \times 0.4475 = 0.038$$

The  $A_{280}$  reading is therefore inflated by 0.038 and needs to be corrected:

$$\text{Corrected } A_{280} \text{ (i.e. to get the contribution from the antibody alone)} = 0.246 - 0.038 = 0.208$$

The corrected  $A_{280}$  value is then used along with the extinction coefficient for antibodies to calculate concentration of conjugate.

$$\text{Ab conjugate concentration} = 10^6 \times 0.208 / 210,000 = 0.9905 \mu\text{M}$$

Since biotin concentration is  $4.416 \mu\text{M}$  and antibody conjugate concentration  $0.9905 \mu\text{M}$ , the average number of biotins per Ab =  $4.416 / 0.9905 = 4.46$

Finally, the recovery is obtained from the concentration of Ab at the start ( $6.7 \mu\text{M}$ ) and concentration of conjugate at end ( $0.9905 \mu\text{M}$ ), and the initial ( $100 \mu\text{L}$ ) and final ( $500 \mu\text{L}$ ) volumes.

$$\text{Recovery} = 100 \times (0.9905 \times 500) / (6.7 \times 100) = 74\%$$

As discussed in Section 5.1, many of the parameters used in the calculations are constants. To simplify the calculations, and to avoid any rounding errors, we would recommend that you set up an Excel template and perform the calculations automatically after inputting the variables i.e.  $A_{280}$  &  $A_{350}$ .

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