A. LB Broth
Materials:
25 g LB broth
1 L distilled water
Procedures:
Add 25g LB broth to 1 litre distilled water
Autoclave

B. LB Agar
Materials:
37 g LB Agar
1 Litre distilled water
Procedures:
Add 37g LB Agar to 1 litre distilled water
Autoclave

C. Glycerol Stocks
Materials:
500µl Filtered glycerol solution (80%)
500µl overnight culture
Procedures:
Add 500µl glycerol (80%) to 1.5ml Glycerol stocks tube
Add 500µl overnight culture in LB
Store at -80°C

Gel Electrophoresis

Materials:
Agarose Powder
TAE buffer
Gel mould
5-10 µL SybrSafe
Gel Tank
8-10 µL DNA ladder
DNA loading dye

Procedures:

1. Prepare 1% w/v solution of agarose powder in 1/10 TAE buffer (e.g. 1.0 g agarose powder in 100 mL buffer) using a conical flask
2. Heat the mixture until agarose is completely dissolved. Do not let the solution boil.
3. Pour the solution into a gel mould
4. Add 5-10 µL SybrSafe to the solution. Make sure there are no bubbles in the solution.
5. Allows the solution to set (approx 15-20 minutes)
6. Transfer the agarose gel to a tank, remove the comb and apply:
   - 8-10 µL of the DNA ladder
   - DNA samples with the corresponding amount of DNA loading dye (6X)
7. Run the gel for 45mins at 100V

www.genomecompiler.com/tips-for-efficient-primer-design/
www.genomeconpiler.com/primer-design-primer-library/

Summary from the website:

1. Primers must be specified 5' to 3', left to right.
2. 18 to 25 nucleotides in length
3. GC content = 40%- 60%
4. 3' ends = C or G, to promote binding (usually 2x, i.e. CC, CG GC GG; called "GC clamp"). GC base pairs have a stronger bond than AT pairs (3 hydrogen bonds vs 2).
5. 3' end, (11 terminal nts) = should be an exact match to the templare DNA (proofreading DNA polymerases will degrade it otherwise).
6. When a restriction site sequence is added onto the 5' end of a primer, an additional 3-6 nts (a leader sequence) are needed to ensure efficient cutting.
7. Avoid runs of 4 or more of any one base, or dinucleotides repeats (ACCCC or ATATATAT); this can cause mispriming.
8. Check for secondary structures: primer dimers self-dimers and hetero dimers, and hairpins.
    - Use IDT oligo analyzer to detect secondary structures and hairpins. https://www.idtdna.com/calc/analyzer
    - The delta G value for dimer analysis should be between 0- -5 kcal/mole for optimal design. More negative values will affect PCR reactions.
    - For hairpins, the melting temperature, Tm should be lower than the annealing temperature and no greater 50 degrees Celsius
9. Primer pairs should have similar Tm with a maximum difference of 5 degrees Celsius.
10. Primers should not be complementary to each other.
11. Use snapgene or ncbiprimer blast(www.ncbi.nlm.nih.gov/tools/primer-blast/) or UCSC in-silico PCR (genome.ucsc.edu) to verify that primer only anneal in one place.
12. Gradient PCR, 2- Step PCR, overlap-extension PCR, Gibson Assenbly/ HiFi, Golden Gate, 3A assembly.

Mini-prep Protocol

Materials:

1. Re-suspension buffer
2. Lysis solution
3. Neutralization solution
4. Wash solution
5. Elution buffer
6. Overnight culture
7. Eppendorf tubes
8. Benchtop centrifuge

Procedures:

1. Prepare overnight culture
2. Start with 2 mL of overnight culture
3. Centrifuge at 12500rpm for 5 mins
4. Discard supernatant and re-suspend with 250µL re-suspension buffer
5. Add 250µL lysis buffer and mix gently by inverting tubes 4-6 times
6. Add 350µL neutralization buffer and invert tubes immediately.
7. Centrifuge at 12500rpm for 5 mins
8. Transfer supernatant into spin-column
9. Add 750µL wash buffer and centrifuge for 1 mins at 12500 rpm.
10. Discard the flow-through and repeat step 9 and 10 again
11. Centrifuge 1 more minute to get rid of ethanol
12. Discard the tubes at the bottom of column and transfer it to Eppendorf tube
13. Add 30-50µl elution buffer and incubate at benchtop for 2 mins
14. Centrifuge for 1 mins
15. Collect the tubes with plasmid in it and store in -20˚C freezer

Overnight Culture Preparation

Materials:

1. 10 ml LB media
2. Falcon Tube
3. Antibiotics (optional depends on strain and plasmid)
4. Inoculation Loops

Procedures:

1. Add 10 ml LB media into Falcon tube
2. Add according antibiotics
3. Use an inoculation loop, pick a colony and touch the culture by dipping
4. Seal the tube and incubate overnight at 37˚C shaking incubator

Transformation

Materials:

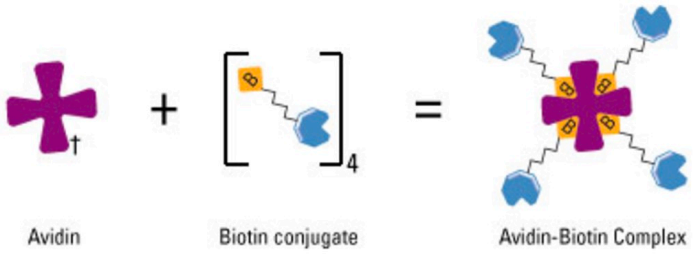
1. Ice box
2. 42˚C water bath
3. 37˚C water bath
4. 37˚C shaking incubator
5. Eppendorf tubes
6. NEB10ß or DH5α competent cells

Procedures:

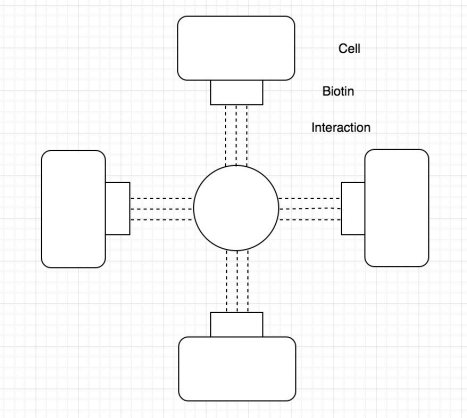
1. Thaw cells on ice
2. Mix 1 - 5 µl of DNA (usually 10 pg - 100 ng) into 20-50 µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.
3. Incubate the competent cell/DNA mixture on ice for 20-30 mins.
4. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 secs is usually ideal, but this varies depending on the competent cells you are using).
5. Put the tubes back on ice for 2 min.
6. Add 250-1,000 µl LB or SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.
7. Plate some or all of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.
8. Incubate plates at 37°C overnight.

20/09/17
Experiment: Cell Adhesion by Biotin-Avidin Conjugation

Experiment Design:
Avidin has a high affinity to biotin, one Avidin molecule can bind up to 4 biotin molecules, which are normally conjugated to an enzyme, antibody or target protein to form an avidin-biotin complex.



By this design, we can apply similar idea to *E.coli* cells. Once cells are biotinylated and biotin molecules are attached to the surface of the cells, the cell-biotin conjugate will be able to bind to avidin once avidin is added. Cell adhesion is achieved.
For tetrameric avidin, the theoretical maximum is 4 moles biotin:1 mole Avidin [1]
There is no way to see how many cells have been biotinylated. Two experiments of different Avidin Biotin-cell ratio are used in the experiment with a control.
Measurements to be discussed.



Materials:
Sulfosuccinimidobiotin, NHS-Biotin Solution, Dimethylformamide(DMF), Ice-cold PBS solution (pH 8.0), *E.coli* pUC 19 Overnight culture , 25˚C Waterbath, Glycine, Avidin and Measurement instruments.

Lab-based Methods:

A. Cell Biotinylation [2]
    1. Wash cells three times with ice-cold PBS (pH 8.0) to remove amine-containing culture media and proteins from the cells. (6000rpm 5 mins each time)
    2. Suspend cells at concentration of OD_{600}= 0.032 in PBS.
    3. 20mM NHS-Biotin solution (4-5mg to 0.5ml DMF).
    4. Add 100µl solution to 1ml of cell suspension and incubate for 30 mins at room temperature.

5. Wash cells with PBS+100mM Glycine to remove excess biotin

B. Biotin-Avidin System

Avidin is very soluble both in water and salt solutions so avidin is going to be dissolved in PBS solution to required molarity.

1. Control: Cells are re-suspended in 100µl of 20mM NHS-Biotin solution and mixed with PBS as control.
2. 1:1: Cells are re-suspended in 100µl of 20mM NHS-Biotin solution and mixed with 100µl 20mM Avidin solution.
3. 1:4: Cells are re-suspended in 100µl of 20mM NHS-Biotin solution and mixed with 25µl 20mM Avidin solution.

Following procedures depend on how measurements are made.

C. Part 3-Cell adhesion measurement

To be discussed

[1] https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/a9275pis.pdf

[2]https://tools.thermofisher.com/content/sfs/manuals/MAN0011206_EZ_NHS_Biotin_Reag_UG.pdf

22/09/17
Bacterial Cell Aggregation by Biotin-Avidin adhesion

Materials:
Biotin: 50 mg ✔
Avidin: 2mg✔
Glycine: >2 g✔
BL21(DE3) pET14b-GFP (Amp): 50mL✔
BL21(DE3) pL4SS-mCherry (Cam): 50mL✔
Autoclaved LB Media: 500 mL✔
Autoclaved Baffled Shaking Flasks with cotton: 2
Fume Hood
Procedures:

A. Solution Preparation (On Wednesday)
    1. 30 mL of 100mM Glycine: 225 mg of glycine in 30mL PBS (Filter glycine solution before use)
    2. Add 2 mg Avidin to 1 mL PBS (0.0312mM Avidin)(3mg left)
    3. Add 8.868 mg Biotin to 1ml DMF (10mM Biotin)
B. Cell Preparation (Ready until Wednesday)
    1. Inoculate both strains onto according plates and incubate at 37˚C overnight✔
    2. Take out 2 plates and put them into 4˚C freezer (Friday) ✔
    3. Pick in total of 5-10 colonies (on one inoculation loop) and inoculate into 10ml LB. ✔
    4. Incubate overnight at 37˚C at 160rpm✔
    5. Prepare 50mL LB with according antibiotics in 250mL baffled shaking flasks (Tuesday)
    6. Inoculate 100µL of overnight culture(Tuesday)
    7. Induce with IPTG when OD_{600} =1 (Tuesday)
    8. Put cultures at room temperature overnight shaking at 160 rpm.
    9. Proceed same procedure with the other strain. Overnight culture OD_{600} >5.0
C. Biotinylation
    1. Wash 20mL of cells three times with ice-cold PBS (pH 8.0) at 5000rpm 5 mins each time
    2. Measure OD600 and adjust to 1
    3. Add 100µl of 10mM NHS-Biotin to 900µL of cells and flick the tube 5-6 times to mix (Repeat for all samples except for the un-biotinylated cells)
    4. Incubate cells in room temperature for at least 1 hour
    5. Wash cells in 1mL Glycine solution to remove excess biotin
    6. Re-suspend cells to OD_{600}=1.0 with 1 mL PBS
D. Adhesion
    For filming aggregation, start with OD_{600}=1.0
    For microscopy and sedimentation with plating, start with OD_{600}=1.0
    Experiment groups: (Repeat same procedure with the other strain)
    Do the experiment on the most fluorescent cells.

Groups	Cells	Biotin	Avidin	DMF	PBS	
Control 1	1mL	-	100µl 0.0312mM	100µl	-	
Control 3	1ml	-	-	100µl	100µl	
Control 2	1mL	100µl 1.0 mM	-	-	100µL	
4:1	1mL	100µl 0.125 mM	100µl 0.0312mM	-	-	
X:1(Excess Biotin)	1mL	100µl 1.0 mM	100µl 0.0312mM	-	-	Excess Biotin
		Control 1	Un-Biotinylated			
		Control 2	Un-Avidinated			
		Control 3	Unlabelled and un-avidinated			

E. Incubation
    1. Incubate all vials of cells at 25˚C water bath for 1 hour.
    2. After incubation, aggregation should have started and measurements should be taken at first time point when they are taken out of the water bath.
    3. Put vials at benchtop for 5 hours. Measurement intervals stated below.
F. Measurements
    1. Film sedimentation
       Use normal phone to focus at the tube, film for 5 hours. (or less)
    2. Natural Sedimentation, plate and count colonies
        1. Let all groups to be settled down. Mark a point for consecutive measurements.
        2. Take out 10 µL of supernatant of the culture FROM SAME POINT. Time interval: 30 mins, 1hour, 2hours, 3hours, 5hours.
        3. Re-suspend 10 µL in 90 µL of LB (x10 dilution)
        4. Plate onto according antibiotics containing plates
        5. Incubate at 37˚C overnight
    3. Cytometric Microscopy
        1. Take 10µL of the initial culture
        2. Observe under wide-field microscope

26/09/17
Bacterial Cell Aggregation by Biotin-Avidin adhesion

Materials:
Nhs Biotin: 50 mg ✔
Avidin: 2mg✔
Glycine: >2 g✔
BL21(DE3) pET14b-GFP (Amp): 50mL✔
BL21(DE3) pL4SS-mCherry (Cam): 50mL✔
Autoclaved LB Media: 500 mL✔
Autoclaved Baffled Shaking Flasks with cotton: 2
Fume Hood
Procedures:

1. **Preparation**
    1. Prepare 50 mL of 100mM Glycine: 375 mg of glycine in 50mL PBS (Filter glycine solution before use)
    2. Add 2 mg Avidin to 1 mL PBS (0.0312mM Avidin)(3mg left)
    3. Add 8.868 mg Biotin to 1ml DMF (10mM Biotin) **– dilute to 1mM?**
    4. Prepare 2x 250mL baffled conical flasks, each containing 50m LB with appropriate antibiotics
    5. Prepare 2x 50mL LB with appropriate antibiotics in 50mL Falcon tubes
    6. Prepare 500mL PBS. Filter sterilize and store in 4˚C fridge.
    7. Prepare an appropriate tube rack and lighting setup for 5 hour video capture of cell aggregation in tubes.

2. **Cell Preparation**
    1. Streak both strains onto plates with appropriate antibiotic and place in static incubate at 37˚C overnight✔
    2. Move plates to 4˚C freezer for overnight storage. Seal with nesco film. ✔
    3. Pick in total of 10 colonies (on one inoculation loop) into 10ml LB with appropriate antibiotic. ✔
    4. Incubate overnight at 37˚C at 160rpm✔
    5. 9am Use 100µL of each culture to inoculate 50m LB with with appropriate antibiotics in 250mL flasks.
    6. Incubate at 37˚C at 160rpm. Monitor OD hourly and add X volume of Y conc. IPTG when OD_{600} =1 then leave incubation to continue overinight.

3. **Biotinylation (for both strains)**
    1. Move 20mL of culture from conical flask to 50mL Falcon tube.
    2. Cf at 5000rpm for 5 mins
    3. Discard supernatant and resuspend pellet in 20mL ice-cold PBS (pH 8.0)
    4. Repeat above 3 steps three times.
    5. Measure OD600 of final PBS / cell solution.
    6. Dilute an aliquot of the PBS / cell solution of the necessary volume to result in an 8mL PBS / cell solution of OD600 = 1.

Biotinylation continued.

7. Transer 900µL of the OD600 = 1 PBS / cell solution to each of eight 1.5mL Eppendorf tubes, labelled A-H.
8. Add the solutions indicated in Table 1 'Step 1 Biotinylation' to each of the tubes, labelled A-H.
9. Incubate all tubes for 1 hour at Room Temp.
10. Cf tubes at XXX RPM. Discard supernatant.
11. Resuspend pellet in 1mL Glycine.
12. Cf tubes at XXX RPM. Discard glycine supernatant.
13. Resuspend pellet in 900µL PBS.

4. Avidin incuabation

14. Add the solutions indicated in Table 2 'Step 2 Avidin incuabation' to each of the tubes. **Take a note of the time.** Gently swirl tubes 3 times.
15. Proceed immediately to aggregation measurement.

5. Aggregation measurements

1. Transfer 500 µL of cell solution gently into a second, labeled tube for video recording.
2. Place these new tubes in an appropriate tube rack and lighting setup for 5 hour video capture of cell aggregation.
3. Place 40x 1.5mL Eppendorf tubes in a rack. Add 90 µL PBS to each tube.
4. Each hour for 5 hours, remove 10µL FROM THE UPPER SURFACE of the culture and add to 90 µL PBS in a separate tube. Transfer what is now a 100µL to a LABELLED LB agar plate, containing appropriate antibiotic, and spread. Place plate in static incubator overnight. Log approx. colony counts the next day.
5. Use the remaining 500 µL of cell solution from step 1. above for microscopy studies with Steffi Frank.

Table 1

	Step 1 Biotinylation					Step 2 Avidin Incubation	
Tube Label	Vol. OD = 1 PBS cell solution	1000µM nhs-Biotin	125µM nhs-Biotin	PBS	DMF	32µM Avidin	PBS
A	900µL				100µl	100µl	
B	900µL				100µl		100µl
C	900µL			100µL		100µl	
D	900µL			100µL			100µl
E	900µL		100µL			100µl	
F	900µL		100µL				100µl
G	900µL	100µL				100µl	
H	900µL	100µL					100µl

Strains Specifications:
BL21(DE3) pET14B-GFP (Amp)
BL21(DE3) pL3SS-mCherry (Cam) incubated on Thursday
Freeze in fridge on Friday over the weekend

For each:

1. Pick 5-10 colonies and inoculate into 10ml LB On Monday
2. Incubate overnight at 37˚C at 160rpm On Monday
3. Prepare 50mL LB with according antibiotics in 250mL baffled shaking flasks On Tuesday
4. Inoculate 500µL of overnight cultures into the flask and incubate at 37˚C at 160rpm On Tuesday
5. Induce with IPTG when OD_{600} =1 On Tuesday
6. Put cultures at room temperature overnight shaking at 160 rpm. On Wednesday

Start Biotinylation experiment

3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Avidin from egg white
BioUltra

Catalog Number **A9275**
Storage Temperature 2–8 °C

CAS RN 1405-69-2

Product Description

In the late 19th century and the 1910's, several reports indicated that feeding large quantities of dried egg white to animals produced a nutritional deficiency.[1,2] Administration of vitamin H, also known as biotin, remedied this deficiency. Eventually, it emerged that this deficiency resulted from the binding of biotin to a protein in egg white.[3,4] This protein was called "avidin", after its "avidity" for biotin.

Avidin is a tetrameric glycoprotein with an approximate molecular mass of 66–67 kDa.[5] It is composed of four subunits with each subunit containing 128 identical amino acid residues and a variable carbohydrate moiety.[5,6] The subunits may vary slightly in molecular mass due to the carbohydrate composition. Only Asp^{17} is glycosylated.[5,7] The carbohydrate moiety can have at least three different carbohydrate structural types.[8] Avidin can be dissociated into subunits under strongly denaturing conditions. Each subunit is separately capable of binding biotin with a dissociation constant (K_d) = 10^{-7} M.[6,9]

The avidin-biotin association constant (K_a = 10^{15} M^{-1}) is one of the strongest affinities known. The complex is stable to 100 °C, and significantly stable to detergents and denaturants.[10] Avidin is stable to ~85 °C without biotin. Biophysical studies of the avidin-biotin complex have implicated particular tryptophan and lysine residues in the biotin-binding site of avidin.[11,12] The crystal structure of avidin has been published.[13] The crystal structure of a deglycosylated form of avidin complexed with biotin has been reported.[14]

Because of the stability of both avidin and biotin, each of these molecules has been used as "labels" for antibodies, fluorescent dyes, proteins, and other molecules of interest to biochemists. Avidin and biotin have each been incorporated into immobilized matrices. (The only way that monomeric avidin can exist is through its attachment to an agarose support.[9])

This product (A9275) is purified using affinity chromatography. It is dialyzed extensively against deionized water before being lyophilized. The basic procedures are modified from literature preparations.[13,15,16] This product is sold by protein content (determined by $E^{1\%}_{280}$).

Unit Definition: One unit will bind 1.0 μg of d-biotin.

Unit activity: ≥10 units per mg protein

Isoelectric point (pI):[8,9] 10

K_d for the avidin-biotin complex:[6] 10^{-15} M (neutral pH)

Binding capacity: For tetrameric avidin, the theoretical maximum is 4 moles biotin:1 mole avidin[6,13] or ~15 μg biotin/mg protein.[14]

Reported fluorescent wavelength:[9]
338 nm (avidin)
328 nm (avidin-biotin complex)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Avidin is very soluble both in water, up to 20 mg/mL,[8] and in salt solutions. Avidin solutions are stable over a wide range of pH and temperatures, particularly when combined with biotin.[6,17] Avidin can be crystallized from ammonium sulfate at >2.5 M at pH 5.[13] Since one tryptophan residue per subunit is involved in the binding site, avidin can be inactivated by oxidizing agents such as ozone, peroxide, or strong light.[11] Solutions should be stored at –20 °C.

Storage/Stability

The avidin-biotin complex is even more heat stable than avidin alone:

- It is only 10% dissociated after 15 minutes at 100 °C.[6] It is not completely dissociated after 60 minutes at 100 °C.
- The complex can be quantitatively dissociated only under autoclaving conditions, e.g. 120 °C, 15 minutes.[15]

When avidin was reduced in the presence of 9 M urea, its biotin-binding activity was unchanged. The protein was denatured and lost biotin-binding activity as the pH was gradually lowered to pH 1. However, when the pH was raised to pH 3, avidin regained native configuration and binding activity. The complex is also extremely stable at high pH, being only 20% ionized even at pH 13.[17]

References

1. Bateman, W.G., *J. Biol. Chem.*, **26(1)**, 263-291 (1916).
2. György, P., *J. Biol. Chem.*, **131(2)**, 733-744 (1939).
3. du Vigneaud, V., *et al.*, *J. Biol. Chem.*, **140(2)**, 643-651 (1941).
4. György, P., *Science*, **93(2420)**, 477-478 (1941).
5. DeLange, R.J., and Huang, T.-S., *J. Biol. Chem.*, **246(3)**, 698-709 (1971).
6. Korpela, J., *Med. Biol.*, **62(1)**, 5-26 (1984).
7. Woolley, D.W., and Longsworth, L.G., *J. Biol. Chem.*, **142(1)**, 285-290 (1942).
8. Bruch, R.C. and White, H.B., III, *Biochemistry*, **21(21)**, 5334-5341 (1982).
9. Green, N.M., *Adv. Protein Chem.*, **29**, 85-133 (1975).
10. Ross, S.E., *et al.*, *BioTechniques*, **4**, 350-354 (1986).
11. Green, N.M., *Biochem. J.*, **89(3)**, 599-609 (1963).
12. Gitlin, G., *et al.*, *Biochem. J.*, **242**, 923-926 (1987).
13. Green, N.M., and Toms, E.J., *Biochem. J.*, **118(1)**, 67-70 (1970).
14. Livnah, O., *et al.*, *Proc. Nat. Acad. Sci. USA*, **90(11)**, 5076-5080 (1993).
15. Green, N.M., *Methods Enzymol.*, **XVIII (Part A)**, 414-417 (1970).
16. Melamed, M.D. and Green, N.M., *Biochem. J.*, **89(3)**, 591-599 (1963).
17. Green, N.M., *Biochem. J.*, **89(3)**, 609-620 (1963).

CKV,GCY,MAM 03/17-1

INSTRUCTIONS

EZ-Link® NHS-Biotin Reagents

20217 21336 21343

0237.4

Number **Description**

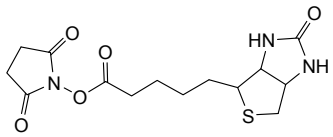
20217 **EZ-Link® NHS-Biotin,** 100 mg, *N*-hydroxysuccinimidobiotin

Molecular Weight: 341.38

Spacer Arm Length: 13.5 Å

Net Mass Added: 226.08

Storage: Upon receipt store desiccated at room temperature.



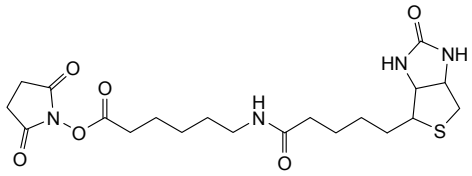
21336 **EZ-Link® NHS-LC-Biotin,** 50 mg, succinimidyl-6-(biotinamido)hexanoate

Molecular Weight: 454.54

Spacer Arm Length: 22.4 Å

Net Mass Added: 339.16

Storage: Upon receipt store desiccated at 4°C. Product is shipped at ambient temperature.



21343 **EZ-Link® NHS-LC-LC-Biotin,** 50 mg, succinimidyl-6-(biotinamido)-6-hexanamido hexanoate

Molecular Weight: 567.70

Spacer Arm Length: 30.5 Å

Net Mass Added: 452.24

Storage: Upon receipt store desiccated at 4°C. Product is shipped at ambient temperature.

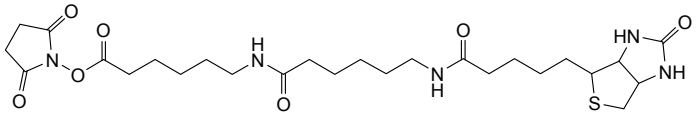


Table of Contents

Introduction

EZ-Link® NHS-Biotin Reagents enable simple and efficient biotin labeling of antibodies, proteins and any other primary amine-containing macromolecules in solution. Differing only in spacer arm lengths, the three reagents offer researchers the possibility of optimizing labeling and detection experiments where steric hindrance of biotin binding is an important factor.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermo.com/pierce

Biotin is a small naturally occurring vitamin that binds with high affinity to avidin and streptavidin proteins. Because it is so small (244 Da), biotin can be conjugated to many proteins without altering their biological activities. Labeled proteins may be purified from unlabeled proteins using immobilized streptavidin and avidin affinity gels (see Related Thermo Scientific Products), and they may be detected easily in ELISA, dot blot or Western blot applications using streptavidin or avidin-conjugated probes.

N-Hydroxysuccinimide (NHS) esters of biotin are the most popular type of biotinylation reagent. NHS-activated biotins react efficiently with primary amino groups (-NH_2) in pH 7-9 buffers to form stable amide bonds. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for labeling with NHS-activated biotin reagents. Several different NHS esters of biotin are available, with varying properties and spacer arm lengths. The three EZ-Link® NHS-Biotin Reagents are not directly water soluble and must be dissolved in organic solvents such as DMSO or DMF before addition to aqueous solutions at the final concentration for the labeling reaction.

Biotinylation of intact cells has emerged as an important tool for studying the expression and regulation of receptors and transporters, differentiation of plasma membrane proteins from those localized to organelle membranes, and distribution of membrane proteins in polarized epithelial cells. Sulfo-NHS-Biotin reagents (see Related Thermo Scientific Products) do not readily permeate cell membranes and are commonly used for specifically labeling the cell surface. By contrast, NHS-Biotin reagents are membrane permeable and may be used to biotinylate proteins inside intact cells. Parallel experiments with NHS- and Sulfo-NHS-Biotin analogs may help to localize particular proteins of interest.

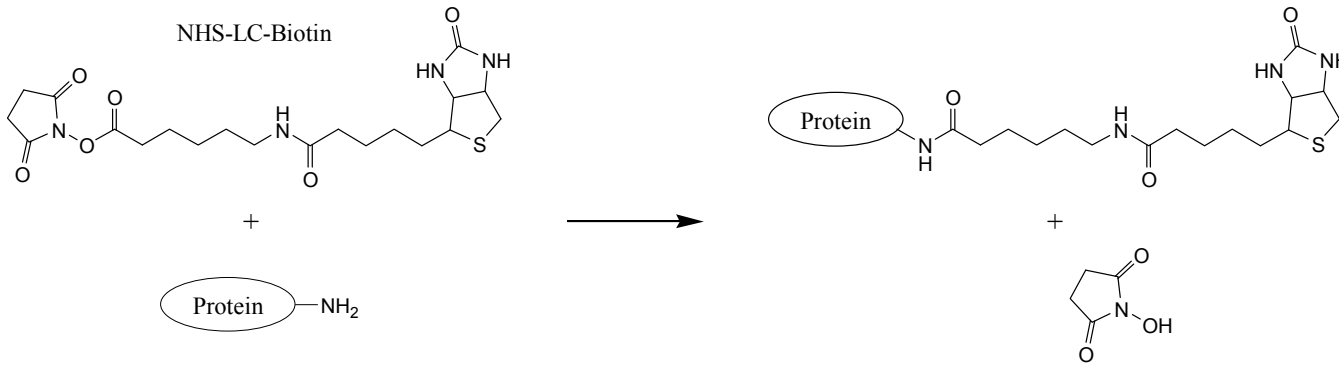


Figure 1. Reaction of NHS-LC-Biotin with primary amine. If drawn to scale, the oval representing the protein would be many times larger than the structures shown in this scheme and would likely contain several amino groups, each of which would be labeled in some proportion of the protein molecules in the reaction. Note that NHS is a leaving group (byproduct) in the reaction; this leaving group as well as any nonreacted biotin reagent is removed during the final desalting step in the procedure.

Important Product Information

- NHS-Biotin reagents are moisture-sensitive. If the vial of reagent has been stored cold, fully equilibrate vial to room temperature before opening to avoid moisture condensation inside the container.
- As directed in the procedure, dissolve the biotin reagent immediately before use. The NHS ester moiety readily hydrolyzes and becomes nonreactive; therefore, weigh and dissolve only a small amount of the reagent at a time, and do not prepare stock solutions for storage. Discard any unused reconstituted reagent.
- Avoid buffers containing primary amines (e.g., Tris or glycine) as these will compete with the reaction (see Figure 1). If necessary, dialyze or otherwise desalt to exchange the protein sample into an amine-free buffer such as phosphate buffered saline (PBS; see Related Thermo Scientific Products).

- When biotinylating proteins in solution, excess non-reacted biotin and reaction byproducts are easily removed by size exclusion using either desalting columns or dialysis (See Additional Information and Related Thermo Scientific Products). A 10 ml desalting column is best suited for processing biotinylation reactions involving 1-10 mg of protein in approximately 0.5-2 ml. For smaller amounts of protein and/or smaller reaction volumes, both the biotinylation reaction and subsequent buffer exchange may be performed in a single Slide-A-Lyzer® MINI Dialysis Unit. For larger reaction volumes than can be processed with a desalting column, either split the sample between two columns or use an appropriate Slide-A-Lyzer® Dialysis Cassette for buffer exchange steps.

Additional Materials Required

- Water-miscible organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF)
- Phosphate Buffered Saline (PBS) or other amine-free buffer having pH 7-8 for use as reaction buffer (see Important Product Information and Related Thermo Scientific Products)
- Desalting columns or dialysis units for buffer exchange (see Important Product Information and Related Thermo Scientific Products)

Procedure for Biotinylating Proteins

The following procedure ordinarily will yield incorporation of 3-5 biotins per molecule of protein. Antibodies, which are large proteins, often will label with ~8-12 biotin molecules per molecule of IgG, especially when greater molar excesses of biotin reagent are used (see Calculations). The molar ratio of biotin reagent to protein may be adjusted to obtain the level of incorporation desired.

A. Calculations

The amount of biotin reagent to use for each reaction depends on the amount of protein to be labeled and its concentration. By using the appropriate molar ratio of biotin to protein, the extent of labeling can be controlled. When labeling more dilute protein solutions, a greater molar fold excess of biotin is necessary to achieve the same results. Generally, for best results use ≥ 12-fold molar excess of biotin for a 10 mg/ml protein solution or ≥ 20-fold molar excess of biotin for a 2 mg/ml protein solution.

1. Calculate millimoles of biotin reagent to add to the reaction for a 20-fold molar excess:

$$\text{ml protein} \times \frac{\text{mg protein}}{\text{ml protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol Biotin}}{\text{mmol protein}} = \text{mmol Biotin}$$

   - 20 = Recommended molar fold excess of biotin for 2 mg/ml protein sample

2. Calculate microliters of 10 mM biotin reagent solution (prepared in Step B.3) to add to the reaction:

$$\text{mmol Biotin} \times \frac{\text{1,000,000 µl}}{\text{L}} \times \frac{\text{L}}{\text{10 mmol}} = \text{µl Biotin}$$

Example Calculation:

For 1 ml of a 2 mg/ml IgG (150,000 MW) solution, ~27 µl of 10 mM biotin reagent will be added.

$$\text{1ml IgG} \times \frac{\text{2 mg IgG}}{\text{1ml IgG}} \times \frac{\text{1mmol IgG}}{\text{150,000 mg IgG}} \times \frac{\text{20 mmol Biotin}}{\text{1mmol IgG}} = \text{0.000266 mmol Biotin}$$

$$\text{0.000266 mmol Biotin} \times \frac{\text{1,000,000 µl}}{\text{L}} \times \frac{\text{L}}{\text{10 mmol}} = \text{26.6 µl Biotin Reagent}$$

B. Biotin Labeling Reaction

1. If the biotin reagent has been stored cold, remove the vial from storage and fully equilibrate it to room temperature before opening in step 3.
2. Dissolve 1-10 mg protein in 0.5-2.0 ml PBS according to the calculation made in section A.

   Note: Protein that is already dissolved in amine-free buffer at pH 7.2-8.0 may be used without buffer exchange or dilution with PBS. Proteins in Tris or other amine-containing buffers must be exchanged into a suitable buffer.
3. Immediately before use, prepare a 10 mM solution of the biotin reagent in an organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF):
   - For NHS-Biotin (Product No. 20217), dissolve 2.0 mg reagent in 590 µl of solvent.
   - For NHS-LC-Biotin (Product No. 21336), dissolve 2.3 mg reagent in 500 µl of solvent.
   - For NHS-LC-LC-Biotin (Product No. 21343), dissolve 2.0 mg reagent in 350 µl of solvent.
4. Add the appropriate volume (see Calculations in section A) of 10 mM biotin reagent solution to the protein solution.
5. Incubate reaction on ice for two hours or at room temperature for 30 minutes.

   Note: Other than the possibility of ordinary protein degradation or microbial growth, there is no harm in reacting longer than the specified time.
6. Protein labeling is complete at this point, and although excess non-reacted and hydrolyzed biotin reagent remains in the solution, it is often possible to perform preliminary tests of the labeled protein by ELISA or Western blot. Once proper function and labeling of the protein has been confirmed, the labeled protein may be purified for optimal performance and stability using desalting or dialysis. If the level of biotin incorporation will be determined using the EZ™ Biotin Quantitation Kit (HABA assay; see Related Thermo Scientific Products), the protein first must be desalted or dialyzed to remove non-reacted biotin.

Procedure for Biotinylating Cells

Many variations of this procedure exist in the literature. Sulfo-NHS-Biotin reagents (see Related Thermo Scientific Products) do not readily permeate cell membranes and are commonly used for specifically labeling the cell surface. By contrast, NHS-Biotin reagents are membrane permeable and may be used to biotinylate proteins inside intact cells. Parallel experiments with NHS- and Sulfo-NHS-Biotin analogs may help to localize particular proteins of interest.

Labeling may be performed on cells in suspension or on adherent cells in culture plates. In the latter situation, diffusion of the NHS-Biotin reagent to all surfaces of the cells will be limited, and labeling will occur predominately on and through the exposed surface. Culture media must be washed from the cells, or amine-containing components will compete and quench the reaction to cell proteins. Using a more concentrated cell suspension is most effective since less biotin reagent will be required in the reaction. Generally, a final concentration of 2-5 mM NHS-Biotin reagent is effective. NHS-Biotin reactions occur more rapidly at higher pH; therefore, pH 8.0 is used in the following example so that labeling can be completed as quickly as possible.

1. Wash cells three times with ice-cold PBS (pH 8.0) to remove amine-containing culture media and proteins from the cells.
2. Suspend cells at a concentration of ~25 × 10^6 cells/ml in PBS (pH 8.0).
3. Prepare a 20 mM solution of NHS-Biotin reagent by dissolving 4-5 mg of reagent per 0.5 ml of water-miscible organic solvent such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF).
4. Add 100 µl of NHS-Biotin reagent solution to each 1 ml of cell suspension (results in ~2 mM biotin reagent).
5. Incubate reaction mixture at room temperature for 30 minutes.

   Note: Longer reaction time may be necessary to ensure significant diffusion of NHS-Biotin reagent into the cells; otherwise, most labeling may occur at the cell surface.
6. Wash cells three times with PBS + 100 mM glycine to quench and remove excess biotin reagent and byproducts.
7. Lyse and/or analyze biotin-labeled cells as required for the research method.

Additional Information

Visit the web site for additional information related to this product, including the Tech Tip procedure titled "Perform labeling and other reactions in Slide-A-Lyzer® Dialysis Cassettes."

Related Thermo Scientific Products

21217	**EZ-Link® Sulfo-NHS-Biotin,** 50 mg, sulfosuccinimidobiotin
21335	**EZ-Link® Sulfo-NHS-LC-Biotin,** 100 mg, sulfosuccinimidyl-6-(biotin-amido)hexanoate
21338	**EZ-Link® Sulfo-NHS-LC-LC-Biotin,** 50 mg, sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate
28372	**BupH™ Phosphate Buffered Saline Packs,** 40 pack, each pack yields 500 ml of 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2 when reconstituted with 500 ml water.
69576	**Slide-A-Lyzer® MINI Dialysis Unit Kit,** for 10-100 µl sample volumes, 10 units plus float
66382, 66807	**Slide-A-Lyzer® Dialysis Cassette Kits,** for 0.5-3 ml and 3-12 ml sample volumes, respectively
89889	**Zeba™ Desalt Spin Columns,** 5 × 2 ml columns, for desalting 200-700 µl samples
89891	**Zeba™ Desalt Spin Columns,** 5 × 5 ml columns, for desalting 500-2,000 µl samples
28005	**EZ™ Biotin Quantitation Kit,** HABA assay kit to determine levels of biotin incorporation
21450, 21440	**EZ-Link® NHS-PEO$_4$-Solid Phase Biotinylation Kit**, one-step antibody labeling and purification
20347	**Immobilized Streptavidin Gel**, 2 ml
20228	**Immobilized Monomeric Avidin Kit**, bind and gently elute biotin-labeled molecules
21126	**Streptavidin, Horseradish Peroxidase Conjugated**, 1 mg

Product References

Bessho, Y., *et al.* (2002). A tRNA aminoacylation system for non-natural amino acids based on a programmable ribozyme. *Nat. Biotechnol.* **20:**723-8.

Bruneau, N., *et al.* (2003). Lectin-like Ox-LDL receptor is expressed in human INT-407 intestinal cells: involvement in the transcytosis of pancreatic bile salt-dependent lipase. *Mol. Biol. Cell* **14:**2861-75.

Fouassier, L., *et al.* (2000). Evidence for Ezrin-Radixin-Moesin-binding phosphoprotein 50 (EBP50) self-association through PDZ-PDZ interactions. *J. Biol. Chem.* **275(32):**25039-45.

Sélo, I., *et al.* (1996). Preferential labeling of α-amino N-terminal groups in peptide by biotin: application to the detection of specific anti-peptide antibodies by enzyme immunoassays. *J. Immunol. Method* **199:**127-38.

Yamauchi, T., *et al.* (2003). Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423:**462-9.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermo.com/pierce