USER GUIDE

Protein-Oligonucleotide Conjugation Kit

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Cat. No. S-9011-1

Storage 2°–8°C — Do Not Freeze.

The Protein-Oligonucleotide Conjugation Kit requires 50–650 µg of protein at a concentration of 1–5 mg/ml in a volume of 50–130 µl. The kit is suitable for conjugating proteins with a molecular weight range of 25,000–950,000 Daltons. A minimum of 15 OD260 of amine-modified oligonucleotide in the 20–100 nucleotide range is required for conjugation. For conjugation of small proteins (< 75 kDa) or long oligos (>50 nucleotides), a minimum of 25 OD260 of amine-modified oligonucleotide is recommended.

Introduction

The Protein-Oligonucleotide Conjugation Kit contains all the necessary reagents and components to perform two conjugation reactions easily and efficiently, each using any protein ≥ 25 kDa and any amino-oligo between 20–100 nucleotides in length. Based on SoluLINK® bioconjugation technology, the conjugation is both efficient and reproducible. The workflow takes around 10 hours total, involving less than 2 hours of hands-on time. First, the amino-oligo is modified with S-4FB and the protein is modified with S-HyNic. Next, the two modified biomolecules are mixed in the presence of a reaction catalyst to form the conjugate. This procedure ensures >95% conversion of protein to conjugate when 3–4 mole equivalents of oligo are added. Because the HyNic-4FB conjugate bond is UV-traceable, the conjugation reaction can be followed spectrophotometrically in real time at 354 nm.

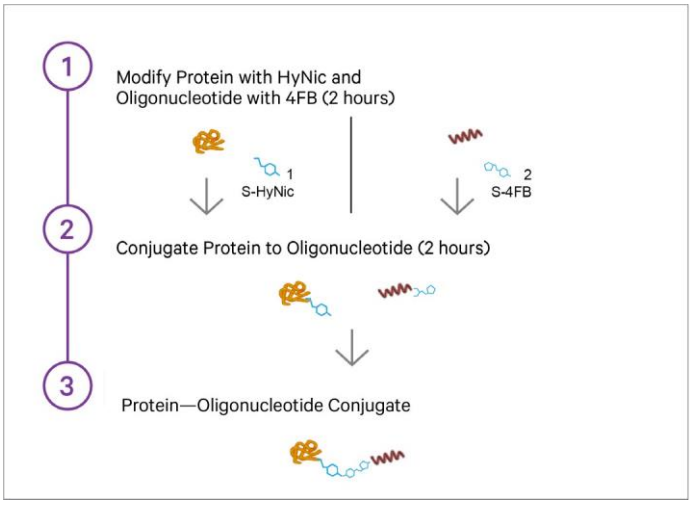

Figure 1. Protein-Oligo Conjugation Kit workflow.

The protein must be at a concentration of ≥ 1 mg/ml for successful conjugation, free of carrier proteins such as BSA or gelatin. The ideal buffer is PBS or another amine-free formulation, however the initial desalting step will remove moderate levels of amine contaminants such as ≤ 100 mM Tris and sodium azide. If the protein contains high concentrations of glycine (≥ 100 mM) it should be desalted into PBS prior to starting. Ensure the protein concentration is between 1.0–5.0 mg/ml and the volume is between 50–130 µl.

The oligonucleotide must be synthesized with an amine on either the 5'- or 3'-terminus. The amino group is modified with S-4FB for conjugation to HyNic-modified protein. Several linker lengths are available for amine incorporation, but the shorter carbon-6 (C6) linker is generally recommended over longer alkyl chains as it is less hydrophobic. At least 15 OD260 units of oligo are required for the modification and conjugation process at a concentration of ≥ 0.3 OD260/µl. HPLC purification of the amino-oligo is highly recommended to remove failure sequences from the synthesis.

Kit Components

Component	Amount
S-HyNic	2 x 1 mg
S-4FB	2 x 1 mg
10X Modification Buffer	1.5 ml
10X Conjugation Buffer	1.5 ml
10X TurboLINK™ Catalyst Buffer	1.5 ml
7kDa MWCO, 0.5 ml Thermo Scientific™ Zeba™ Column	10
Anhydrous DMF	1.5 ml
2-Hydrazinopyridine (2-HP)	0.5 ml
2-Sulfobenzaldehyde (2-SB)	0.5 ml
7kDa MWCO, 2 ml Zeba Column	2
10X PBS	1.5 ml
Oligo Resuspension Solution	1.0 ml
2 ml Collection Tube	12

Protocol

Before using the Protein-Oligonucleotide Conjugation Kit, remove from refrigerated storage and allow components to warm up to room temperature for at least 30 minutes. If precipitate is present in any of the 10X buffers, re-dissolve them by warming at 37°C with periodic vortexing. Ensure all precipitate is completely dissolved before proceeding. The anhydrous DMF contains molecular sieves to absorb moisture; these appear as small beads at the bottom of the tube and are not precipitate.

The Modification and Conjugation Buffers in this kit are supplied as 10X stock solutions. Before beginning, create a 1X working solution of each buffer by mixing 500 µl of 10X stock with 4.5 ml of ultrapure water per conjugation reaction to be performed.

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A minimum of 15 OD_{260} units of amino-oligo is required for the modification and conjugation process. The certificate of analysis from the oligo vendor will state the amount of oligo delivered in OD_{260}. If the tube contains between 15 and 25 OD_{260} of oligo, add 50 µl of Oligo Resuspension Solution to the tube. If the tube contains more than 25 OD_{260} units of oligo, add a sufficient volume of Oligo Resuspension Solution to create a 0.5 OD_{260} /µl solution. For example, if there are 45 OD_{260} units of oligo, add 90 µl of Oligo Resuspension Solution to create a 0.5 OD_{260} /µl solution. Vortex on low speed periodically over several minutes to ensure the oligo pellet is completely dissolved.

A. Measure amino-oligo concentration

The oligo concentration can be measured using either a conventional UV-Vis spectrophotometer or a micro-volume spectrophotometer (e.g., NanoDrop™). When using a conventional spectrophotometer, a 1-cm pathlength quartz or UV-transparent plastic cuvette is required.

Conventional UV-Vis Spectrophotometer method

1. In a microcentrifuge tube, prepare a 1:500 dilution of the oligo by transferring 2.0 µl of oligo into 998 µl of water and vortexing to mix.
2. Blank the spectrophotometer at 260 nm with water.
3. Measure the 260 nm absorbance of the 1:500 oligo solution. Record the A_{260} value.
4. Divide this number by 2 to calculate the OD_{260} /µl concentration of the stock oligo solution.
5. Multiply the OD_{260} /µl value calculated above by the volume of oligo solution to determine the total OD_{260} units available (do not enter this information into the calculator at this point).

   Important: If less than 15 OD_{260} units are recovered after resuspension, obtain additional amino-oligo.

NanoDrop spectrophotometer method (models ND-1000 and ND-2000)

1. In a microcentrifuge tube, prepare a 1:200 dilution of the oligo by transferring 2.0 µl of oligo into 398 µl of water and vortexing to mix.
2. Select the “Nucleic Acid” menu option on the NanoDrop and initialize the instrument using water (NanoDrop ND-1000 only).
3. Blank the instrument with 2.0 µl of water.
4. Measure the 260 nm absorbance of the 1:200 oligo solution as displayed in the 10-mm pathlength window. Record the A_{260} value.
5. Divide this number by 5 to calculate the OD_{260}/µl concentration of the stock oligo solution.
6. Multiply the OD_{260}/µl value calculated above by the volume of oligo solution to determine the total OD_{260} units available (do not enter this information into the calculator at this point).

   Important: If less than 15 OD_{260} units are recovered after resuspension, obtain additional amino-oligo.

B. Prepare two 0.5 ml Zeba columns

1. Prepare two 0.5 ml Zeba columns (red cap) by loosening the caps one-half turn, twisting off the bottom closures, and placing them in 2 ml collection tubes.
2. Using a lab marker, place a vertical line on the outside of each column. Ensure the lines face outward (away from the center of the rotor) in this and all subsequent steps.
3. Centrifuge the columns at 1,500 x g for 1 minute to remove storage buffer.

   Important: Ensure the centrifuge is set to “g” or RCF rather than RPM in all centrifugation steps.
4. Discard the storage buffer and place the spin columns back into the same tubes.
5. Add 300 µl of 1X Modification Buffer to the top of each resin bed and centrifuge at 1,500 x g for 1 minute.
6. Repeat steps 4 and 5 two additional times.
7. The columns are now ready for sample loading.

C. Desalt the protein and amino-oligo

1. Place the equilibrated Zeba columns in new 2 ml collection tubes.
2. Label one of the columns “protein” and the other column “oligo.”
3. Remove the cap from the column labeled “protein” and slowly apply between 50–130 µl of protein sample onto the center of the compact resin bed. Avoid contact with the tube walls — the sample must channel down through the resin itself.
4. Replace the cap on the protein sample and loosen one-half turn.
5. Remove the cap from the column labeled “oligo” and slowly apply between 50–100 µl of oligo sample onto the center of the compact resin bed. Avoid contact with the tube walls — the sample must channel down through the resin itself.
6. Replace the cap on the oligo sample and loosen one-half turn.
7. Centrifuge both columns at 1,500 x g for 2 minutes to collect the desalted samples in the collection tubes.
8. Transfer each sample to a new microcentrifuge tube labeled “desalted protein” or “desalted oligo” while measuring the volume with a pipet.

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D. Modify the amino-oligo with S-4FB

On worksheet 1 of the Protein–Oligonucleotide Conjugation Calculator, enter the required information in the light green input fields of section 1. Oligo-specific information such as the molar extinction coefficient, molecular weight, and nanomoles/OD_{260} can be found on the oligo vendor's certificate of analysis.

1. Measure the amino-oligo concentration after desalting into 1X Modification Buffer by following the procedure in step A.
2. Enter the calculated OD_{260}/µl oligo concentration into section 2 of the calculator, along with the volume of oligo obtained from the desalting step.

   Important: A minimum of 10 OD_{260} is required to continue. If the oligo OD_{260} available is insufficient to proceed, obtain additional amino-oligo.
3. Add the calculated volume of anhydrous DMF from section 3 of the calculator to the oligo solution and mix well. This volume is equal to one-half of the oligo volume.
4. Briefly centrifuge a 1.0 mg vial of S-4FB at 15,000 x g to ensure that all the material is at the bottom.
5. Dissolve the S-4FB in 40 µl of anhydrous DMF to create a 25 mg/ml solution. The solution may need to be vortexed and pipetted up and down several times to dissolve completely. Briefly spin the solution to the bottom of the tube.
6. Add the calculated volume of S-4FB in anhydrous DMF from section 3 to the amino-oligo and vortex to mix. Do not centrifuge the reaction mixture until the reaction is complete.
7. Incubate the modification reaction at room temperature for 1.5–2.0 hours.

   Note: While the oligo is being modified with S-4FB, the protein may be modified with S-HyNic.

E. Desalt the 4FB-modified oligo

1. Five minutes prior to the end of the 4FB-oligo modification reaction, equilibrate two 0.5 ml Zeba columns (red cap) in 1X Conjugation Buffer instead of Modification Buffer as described in step B.
2. Centrifuge the 4FB-oligo modification reaction at 10,000 x g to pellet any insoluble reaction byproducts that may be present.
3. Label one of the equilibrated Zeba columns #1 and the other #2.
4. Load the clear supernatant from the 4FB-oligo modification reaction onto column #1 and centrifuge as described in step C. Use a microcentrifuge tube containing water as a balance.

   Important: Leave column #2 on the bench during this step—it must not be used as a balance.
5. Immediately transfer the entire eluate from the column #1 collection tube to the top of column #2 and centrifuge as described in step C. This "double-desalting" will ensure that all traces of un-incorporated 4FB are removed from the oligo.
6. Transfer the desalted 4FB-oligo to a labeled microcentrifuge tube while measuring the volume with a pipet.

F. Measure the 4FB-oligo concentration and calculate yield

1. Gently vortex the 4FB-oligo solution to mix.
2. Measure the oligo concentration as described in step A.
3. Enter the calculated 4FB-oligo concentration into section 4 of the Protein–Oligonucleotide Conjugation Calculator.
4. Multiply the concentration (OD_{260} /µl) by the volume (µl) to determine the 4FB-oligo yield.

The 4FB-oligonucleotide information may be entered into worksheet 3 of the Protein–Oligonucleotide Conjugation Calculator, along with the protein molecular weight, estimated protein concentration and desired mass of protein to be conjugated to determine if there is enough oligo. For longer oligos and/or smaller proteins, the amount of protein to be conjugated may be limited by the amount of 4FB-oligo available.

G. Measure the oligo 4FB molar substitution ratio (MSR)

The 4FB MSR assay quantifies the percentage of amino-oligo converted to 4FB-oligo. This assay ensures that the oligo is 4FB-modified and properly buffer exchanged.

1. Prepare a 2-hydrazinopyridine (2-HP) blank by adding 2.0 µl of 1X Conjugation Buffer to 18.0 µl of 2-HP reagent in a microcentrifuge tube. Label this tube "2-HP Blank."
2. Prepare a 4FB-oligo MSR sample by adding 2.0 µl of 4FB-modified oligo to 18.0 µl of 2-HP reagent in a separate microcentrifuge tube. Label this tube "4FB-Oligo MSR."
3. Vortex both solutions to mix, then briefly spin the tubes to collect the contents at the bottom.
4. Incubate both tubes at 37°C for 60 minutes.
5. After the incubation period, centrifuge both tubes at 15,000 x g for 15 seconds to collect condensation from the lids. Vortex to mix.

   Use the appropriate instructions below depending on the type of spectrophotometer available.

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Conventional UV-Vis spectrophotometer 4FB MSR method (≤ 200 µl micro-cuvette)

1. Prepare a 1:10 dilution of the 2-HP Blank and 4FB-Oligo MSR reaction by adding 180 µl of water to each tube.
2. Using a 1-cm pathlength micro-cuvette, blank the spectrophotometer at 360 nm with the diluted 2-HP Blank.
3. Measure the 360 nm absorbance of the diluted 4FB-Oligo MSR reaction.
4. Enter this value directly into section 5 of the Protein–Oligonucleotide Conjugation Calculator.

NanoDrop 4FB MSR method

1. Launch the NanoDrop software and select the UV-Vis option.
2. Initialize the instrument with 2 µl of water if necessary (NanoDrop ND-1000 only).
3. Blank the instrument with 2 µl of 2-HP Blank.
4. Set the λ1 absorbance wavelength to 360 nm.
5. Load 2 µl of the 4FB-Oligo MSR reaction and read the 360 nm absorbance. This value should be a 1-mm pathlength absorbance rather than a 1-cm pathlength.
6. Enter the 1-mm absorbance value into section 5 of the Protein–Oligonucleotide Conjugation Calculator.

   Ensure the 4FB-oligo concentration has been entered in section 4, and the oligo information has been entered in section 1. The calculator will display the oligo 4FB MSR. An MSR value of ≥ 0.5 (50%) is required for conjugation.

Guidelines for Modifying Proteins with S-HyNic

The number of linker groups incorporated per protein molecule referred to as the HyNic molar substitution ratio (MSR). The MSR obtained after a modification reaction with S-HyNic is a function of several variables that include protein concentration, number of available amino groups on the protein (often related to molecular weight), and the number of excess linker equivalents added (e.g. 10X, 15X or 20X).

As the protein concentration and number of linker equivalents are increased the MSR increases. Caution is recommended, however, as over-modification can change the isoelectric point of the protein and result in precipitation or loss of biological activity. This is especially important with proteins <50 kDa. Table 1 presents the HyNic MSR obtained when modifying an antibody (150 kDa) with three different mole ratios of S-HyNic at three different protein concentrations.

In general, at a protein concentration of approximately 2.5 mg/ml, 10 mole equivalents of S-HyNic are typically used to modify proteins less than 100 kDa, and 15 mole equivalents are used to modify proteins greater than 100 kDa. An MSR of 4–8 is recommended for proteins greater than 100 kDa, and 2–4 for proteins equal to or less than 100 kDa.

Protein Concentration	5X HyNic	10X HyNic	20X HyNic
1.0 mg/ml	1.0	1.4	3.0
2.5 mg/ml	3.2	5.3	7.9
5.0 mg/ml	4.9	6.6	7.8

Table 1. Antibody HyNic MSR values at various input equivalents and protein concentrations.

H. Modify the protein with S-HyNic

1. On worksheet 2 of the Protein–Oligonucleotide Conjugation Calculator, enter the protein name, molecular weight, concentration, and volume of protein to be modified in the protein information section.
2. Enter the mole equivalents of S-HyNic linker desired to modify the protein.
3. The volume of anhydrous DMF required to dissolve the 1.0 mg aliquot of S-HyNic will be displayed in the orange field.

   Note: If the volume of anhydrous DMF required is more than 700 µl, a dilution of the HyNic reagent will be required. For example, if the volume of anhydrous DMF required is 5,857 µl, the following dilutions would be made:

   (a) Make a 58.57X stock solution by dissolving S-HyNic pellet in 100 µl of anhydrous DMF (5,857 µl /100 µl = 58.57).

   (b) Next, make a 5.857X S-HyNic solution by adding 10 µl of 58.57X S-HyNic solution into 90 µl of anhydrous DMF (1:10 dilution).

   (c) Finally, make a 1X S-HyNic solution by adding 10 µl of 5.857X S-HyNic solution into 48.57 µl of anhydrous DMF (1:5.857 dilution).
4. Add the calculated volume of S-HyNic to the protein solution and immediately pipet up and down, then gently vortex or flick the tube to mix.

   Note: 2.0 µl of S-HyNic is required for proteins >50 kDa, and 3.0 µl is required for proteins ≤ 50 kDa.
5. Incubate the reaction at room temperature for 2.0 hours.

I. Desalt the protein

1. Five minutes prior to the end of the HyNic-protein modification reaction, equilibrate one 0.5 ml Zeba column (red cap) in 1X Conjugation Buffer instead of Modification Buffer as described in step B.

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2. Desalt the protein as described in step C.
3. Transfer the desalted protein to a microcentrifuge tube labeled "HyNic-modified protein."

J. Determine protein concentration and HyNic MSR

After desalting the HyNic-protein into 1X Conjugation Buffer, determine the protein concentration using a BCA or Bradford assay. The protein concentration must not be determined by absorbance at 280 nm due to interference at this wavelength by the incorporated HyNic linkers. Ideally the protein standard curve should be comprised of the same protein being conjugated for the most accurate results. Enter the HyNic-modified protein concentration into worksheet 2 of the HyNic MSR Calculator.

After the HyNic-protein concentration has been determined, measure the HyNic MSR as follows.

1. Prepare a blank by adding 2.0 µl of 1X Conjugation Buffer into 18.0 µl of 2-sulfobenzaldehyde (2-SB) solution in a microcentrifuge tube. Label this tube "MSR Blank."
2. Transfer 2.0 µl of HyNic-modified protein into a separate 1.5 ml microcentrifuge tube containing 18.0 µl of 2-sulfobenzaldehyde solution. Label this tube "MSR Reaction."
3. Vortex both tubes to mix, then incubate at 37°C for 1 hour.
4. Briefly centrifuge the tubes at 10,000 x g to collect condensate from the cap.
5. Vortex both samples before reading the 348 nm absorbance by one of the following methods:

<u>Conventional UV-Vis spectrophotometer MSR method (≤ 200 µl micro-cuvette)</u>

1. Prepare a 1:10 dilution of the MSR Blank and MSR Reaction by adding 180 µl of water to each tube. Vortex to mix.
2. Blank a spectrophotometer at 348 nm with the diluted MSR Blank.
3. Record the 348 nm absorbance of the MSR Reaction.
4. Multiply the absorbance value by 10 to account for the 10-fold dilution, then enter this value into worksheet 2 of the HyNic MSR Calculator.

<u>NanoDrop spectrophotometer MSR method</u>

1. Launch the NanoDrop software and select the UV-Vis menu option.
2. Initialize the instrument with 2 µl of water if necessary (NanoDrop ND-1000 only).
3. Blank the instrument with 2 µl of the MSR Blank.
4. Set the λ1 wavelength to 348 nm.
5. Place 2 µl of the MSR Reaction on the pedestal and click the "Measure" icon. The 1-mm A348 will be displayed.
6. Multiply this number by 10 to convert from a 1-mm to a 1-cm pathlength, then enter this value into worksheet 2 of the HyNic MSR Calculator.

The HyNic MSR Calculator will display the MSR value. An MSR of 4–8 is recommended for proteins greater than 100 kDa, and an MSR of 2–4 is recommended for proteins equal to or less than 100 kDa.

K. Conjugate the HyNic-modified protein to the 4FB-modified oligo

On worksheet 3 of the Protein–Oligonucleotide Conjugation Calculator input the protein information into the light green input fields. Next, enter the oligonucleotide information into the light green input fields. The calculator will output the volume of HyNic-modified protein to mix with the 4FB-modified oligo.

1. Ensure all information in the light green input fields is correct on worksheet 3.
2. Transfer the required volume of HyNic-modified protein to a microcentrifuge tube.
3. Add the required volume of 4FB-oligo to the HyNic-protein. Pipet up and down to mix.

   Note: If the volume of oligo required is less than 2 or 3 µl, a 10-fold dilution of the oligo can be made in 1X Conjugation Buffer and 10X the volume used for more accurate pipetting.
4. Add the required volume of 10X TurboLINK Catalyst Buffer to the conjugation reaction. Pipet up and down, then gently vortex or flick the tube to mix.

   Note: Add the 10X TurboLINK Buffer directly to the conjugation reaction–it does not require dilution to 1X prior to use.
5. Incubate at room temperature for 2–3 hours, or at 4°C overnight.
6. The conjugate is now ready for purification by column chromatography, if desired, or for desalting into storage buffer. Size exclusion chromatography (SEC), anion-exchange chromatography (AX-FPLC), or affinity chromatography may be used for purification, depending on the oligo length and protein molecular weight. It is not recommended to leave the conjugate in 1X Conjugation Buffer for extended periods (> 2 days).

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L. Prepare a 0.5 ml or 2 ml Zeba column for desalting the conjugate

10X PBS, pH 7.20, is included in this kit for desalting the conjugate. Prepare 1X PBS by adding 0.5 ml of 10X PBS to 4.5 ml of ultrapure water. Two different sizes of Zeba desalting columns are included for desalting the protein-oligo conjugate into PBS. For conjugate volumes up to 130 µl, a 0.5 ml Zeba column (red cap) should be used. Follow the procedure in step B to equilibrate a single 0.5 ml Zeba column in 1X PBS. Add the conjugate and elute the sample as described in step C. To desalt larger volumes of conjugate (from 130 µl to 700 µl), follow the 2 ml Zeba column procedure below.

1. Prepare a 2 ml Zeba spin column by loosening the cap one-half turn, twisting off the bottom closure, and placing it in an empty 15 ml conical tube (not provided).
2. Using a lab marker, place a vertical line on the outside of the column. Ensure this line faces outward (away from the center of the rotor) in this and all subsequent steps.
3. Centrifuge the column at 1,000 x g for 2 minutes to remove storage buffer.

   Important: Ensure the centrifuge is set to “g” or RCF rather than RPM in all centrifugation steps.
4. Discard the buffer and place the spin column back into the same tube.
5. Add 1.0 ml of 1X PBS to the top of the resin bed and centrifuge at 1,000 x g for 2 minutes.
6. Repeat steps 4 and 5 two additional times.
7. The column is now ready for conjugate loading.

M. Desalt the conjugate into storage buffer

1. Place the equilibrated Zeba column into a new 15 ml conical tube.
2. Remove the cap and slowly apply 130–700 µl of conjugate onto the center of the compact resin bed.

   Important: For conjugate volumes less than 500 µl, apply 200 µl of PBS to the top of the resin after the conjugate has been completely absorbed. This buffer “stacker” will ensure the conjugate is fully recovered from the column.
3. Centrifuge at 1,000 x g for 2 minutes to collect the desalted conjugate in the conical tube.
4. Transfer the desalted conjugate to a labeled storage tube (not provided).

N. Determine concentration of protein-oligo conjugate

The protein-oligo conjugate concentration may be determined using a BCA or Bradford protein assay. Absorbance at 280 nm must not be used at this step to determine concentration due to the absorbance from the linkers and the attached oligonucleotides. For long-term storage add a bacteriostat such as 0.05% sodium azide or 0.01% thimerosal. Store the conjugate at 2–8°C.

Application Notes

Bradford Assay Protocol

BCA Assay Protocol

Troubleshooting Guide

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