Versalinx® Experimenter's Kit
Protein Modification Reagents

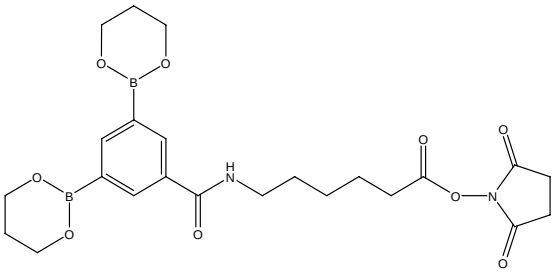
Please note: Kit also contains Versalinx® SHA Agarose Chromatography Medium (50592)

Introduction

The Versalinx Experimenter's Kit contains four Protein Modification Reagents (i.e. Versalinx Amine Modifying Reagent, Carbohydrate Modifying Reagent, Disulfide Forming Reagent and Sulfhydryl Alkylating Reagent) and SHA-Modified Agarose Chromatography Media. The basis of the system is the specific interaction between these two non-biological molecules, phenyl(di)boronic acid (PDBA) and salicylhydroxamic acid (SHA). PDBA can be covalently attached to a protein or peptide using one of the Protein Modification Reagents, the choice of the protein modification reagent will depend on the structure of the protein.

Amine Modifying Reagent

Using our Versalinx® Amine Modifying Reagent (PDBA-X-NHS), PDBA can be selectively conjugated to free amines on lysine residues of a protein.



2,5-dioxopyrrolidinyl 6-{[3,5-di(1,3,2-dioxaboran-2-yl) phenyl]carbonylamino}hexanoate (PDBA-X-NHS)

Reagents and Solutions not Provided

- Protein of interest
- 0.1 M sodium bicarbonate, pH 8.0
- Anhydrous N, N-Dimethylformamide (DMF) (Sigma-Aldrich Part # 22,705-6 or equivalent) OR anhydrous Dimethyl Sulfoxide (DMSO) (Aldrich Part # 34869 or equivalent)
- Dialysis tubing or appropriate molecular weight cut off size exclusion column or ultrafiltration tube.

Amine Modifying Reagent Procedure

1. Prepare a solution of the protein of interest (protein ligand) (1-10 mg/ml) in 0.1 M sodium bicarbonate buffer, pH 8.0. If the protein is already in solution, perform a buffer exchange to the above buffer, either through dialysis, using a gel filtration column, or ultrafiltration tube.
2. Measure the UV absorbance of the protein ligand solution. Using the literature value for the absorbitvity and molecular weight of the protein, calculate the concentration of the stock solution and the moles of protein to be conjugated.
3. Prepare a 100 mM solution of PDBA-X-NHS in anhydrous N,N-Dimethylformamide or anhydrous Dimethyl Sulfoxide. Vortex to dissolve.
4. Decide on the desired molar input ratio of PDBA-X-NHS to protein ligand. A 10:1 molar input ratio is a good starting point. Use the following calculation to determine how many microliters of a 100 mM PDBA-X-NHS solution to add to the protein ligand solution. *The final concentration of DMF/DMSO in the conjugation reaction should be below 10%(v/v)*

$$\mu l \text{ PDBA-X-NHS} = (a)(b)(c)\left(\frac{1}{d}\right)\left(\frac{1}{e}\right)10^6$$

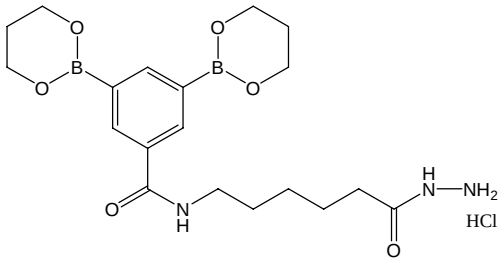
where:
a = desired molar input ratio (PDBA:protein ligand)
b = concentration of protein ligand in mg/ml
c = ml of protein ligand solution to be conjugated
d = MW of protein ligand
e = concentration of PDBA-X-NHS, mM

5. Add the calculated volume of 100 mM PDBA-X-NHS to the protein ligand solution and incubate on ice for one hour.

6. Optional: the reaction mixture can be purified of unwanted by-products and reactants through dialysis, passing through a 500 MWCO size exclusion column, or ultracentrifugation with the appropriate filter.

Carbohydrate Modifying Reagent

Using our Versalinx® Carbohydrate Modifying Reagent (PDBA-X-Hydrazide), PDBA can be selectively conjugated to oxidized sialic acid residues or galactose residues of glycoproteins. Alternatively, a glycoprotein can be oxidized to make a number of aldehyde sites available for hydrazide conjugation. A 10 mM sodium periodate solution can be used to oxidize carbohydrate hydroxyls to aldehydes. A 1 mM sodium periodate solution oxidizes mostly sialic acid residues. Galactose residues can be oxidized using galactose oxidase after treatment with neuraminidase.



6-(N-(3,5-Bis(dihydroxyboryl))benzoyl)aminohexanoic acid hydrazide hydrogen chloride (PDBA-X-Hydrazide HCl)

Cambrex Bio Science Rockland, Inc.
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Reagents and Solutions not Provided

- Glycoprotein of interest
- 350 mM sodium periodate
- Sodium acetate
- Sodium chloride
- 6 M hydrochloric acid
- Sodium bicarbonate
- 0.4 M sodium sulfite
- Anhydrous N, N-Dimethylformamide (DMF) (Aldrich Part # 22,705-6 or equivalent) OR anhydrous Dimethyl Sulfoxide (DMSO) (Aldrich Part # 27,043-1 or equivalent)
- Dialysis tubing or appropriate MWCO size exclusion column or ultrafiltration tube.

Carbohydrate Modifying Reagent Procedure

1. Prepare a buffer solution consisting of 0.1 M sodium acetate plus 0.1 M sodium chloride. Adjust the pH to 5.5 using 6 M HCl.
2. Dissolve 0.1g of the glycoprotein in 10 ml of the buffer prepared in Step 1. If the protein is already in solution, perform a buffer exchange to the above buffer, either through dialysis, using a gel filtration column, or ultrafiltration tube.
3. Measure the UV absorbance of a diluted aliquot of the protein solution. Using the literature value for the absorbtivity and molecular weight of the protein, calculate the concentration of the stock solution and the moles of protein to be conjugated.
4. Proceed with the oxidation as follows. Prepare a 350 mM solution of sodium periodate in water. Add the sodium periodate to your protein solution to a final concentration of 10 mM. Allow to react on ice, in the dark, for 30 minutes. Quench the glycoprotein oxidation by the addition of freshly prepared 0.4 M sodium sulfite to a final concentration of 20 mM. Shake well to mix. The quenching reaction occurs instantaneously.
5. Prepare a 200 mM solution of PDBA-X-Hydrazide in anhydrous N,N-Dimethylformamide or anhydrous Dimethyl Sulfoxide. Vortex to dissolve. Dilute to 100 mM using the buffer prepared in Step 1. Vortex to dissolve.
6. Decide on the desired molar input ratio of PDBA-X-Hydrazide to protein. A 10:1 molar input ratio is a good starting point. Use the following calculation to determine how many microliters of a 100 mM PDBA-X-Hydrazide solution to add to the protein solution.

   The final concentration of DMF/DMSO in the conjugation reaction should be below 10%(v/v)

   µl PDBA-X-Hydrazide = $(a)(b)(c)\left(\frac{1}{d}\right)\left(\frac{1}{e}\right)10^6$

   where:
   - a = desired molar input ratio (PDBA:protein ligand)
   - b = concentration of protein ligand in mg/ml
   - c = ml of protein ligand solution to be conjugated
   - d = MW of protein ligand
   - e = concentration of PDBA-X-hydrazide, mM
7. Add the calculated volume of 100 mM PDBA-X-Hydrazide to the oxidized glycoprotein solution and incubate on ice for 4 hours.

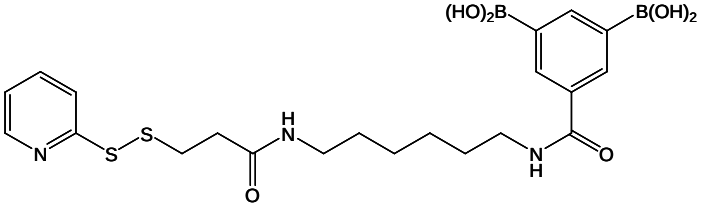
Optional: the reaction mixture can be purified of unwanted by-products and reactants through dialysis, passing through a 500 MWCO size exclusion column, or ultracentrifugation with the appropriate filter, using a buffer of your choice.

Disulfide Forming Reagent

Using our Versalinx® Disulfide Forming Reagent (PDBA-X-dithiopyridyl), PDBA can be selectively conjugated to sulfhydryl residues on proteins, as well as other sulfhydryl groups. It contains an eleven atom spacer between the sulfhydryl-reactive dithiopyridyl functionality and the PDBA moiety. The reaction produces a new mixed disulfide between the PDBA moiety and the sulfhydryl on the biological macromolecule, and releases pyridine-2-thione.

Typically, PDBA-X-dithiopyridyl is added as a concentrated solution in methanol, methyl sulfoxide, or N,N-dimethylformamide to an aqueous solution of the sulfhydryl-containing biological macromolecule. Disulfide-reducing agents such as 2-mercaptoethanol, 2-mercaptoethylamine, and dithiothreitol must be excluded from the reaction mixture, since this will non-productively react with the dithiopyridyl. EDTA should be added to the reaction mixture to retard the re-oxidation of reduced disulfides.

This protocol describes the general approach to the modification of proteins not naturally containing free sulfhydryls. The degree of modification is controlled both by the number of free sulfhydryls generated on the protein ligand as well as by the amount of PDBA-X-dithiopyridyl used for the modification. Titration experiments may be necessary to determine the optimal degree of modification for a given protein.



N-(6-(3-(2-Pyridyldithio)propionyl)amino)hexyl-3,5-bis (dihydroxyboryl)benzamide (PDBA-X-PDP)

Reagents and Solutions not Provided

- Protein of interest
- Sodium citrate
- Citric acid
- EDTA
- Dithiothreitol
- Anhydrous N, N-Dimethylformamide (DMF) (Aldrich Part # 22,705-6 or equivalent) OR anhydrous Dimethyl Sulfoxide (DMSO) (Aldrich Part # 27,043-1 or equivalent)
- Dialysis tubing or appropriate MWCO size exclusion column or ultrafiltration tube.

Disulfide Forming Reagent Procedure

1. Prepare a buffer solution consisting of 0.1 M citrate by combining 95 ml 0.1 M sodium citrate plus 5 ml 0.1 m citric acid. The pH should be nearly 6.5, make adjustments by adding more of the appropriate solution.
2. Add EDTA to the citrate buffer to a final concentration of 1 mM using 0.1 M EDTA in 1 M NaOH
3. Dissolve 50 mg of the protein in 10 ml of the citrate, EDTA buffer. If the protein is already in solution, perform a buffer exchange to the above buffer, either through dialysis, using a gel filtration column, or ultrafiltration tube.
4. Measure the UV absorbance of a diluted aliquot of the protein solution. Using the literature value for the absorbtivity and molecular weight of the protein, calculate the concentration of the stock solution and the moles of protein to be conjugated.
5. Proceed with the reduction as follows. To a 5 ml aliquot of the protein solution, add DTT to 25 mM. Mix gently to avoid oxidation. Incubate at 37^{o}C for an hour. DO NOT SHAKE
6. Remove the DTT by dialysis or filtration, into the 0.1 M Citrate, 1 mM EDTA buffer.
7. Assuming all protein is recovered, calculate the new protein concentration based on the new volume.
8. Prepare a 100 mM solution of PDBA-X-dithiopyridyl in anhydrous N,N-Dimethylformamide or anhydrous Dimethyl Sulfoxide. Vortex to dissolve.
9. Decide on the desired molar input ratio of PDBA-X-dithiopyridyl to protein. A 10:1 molar input ratio is a good starting point. Use the following calculation to determine how many microliters of a 100 mM PDBA-X-dithiopyridyl solution to add to the protein solution.

   The final concentration of DMF/DMSO in the conjugation reaction should be below 10%(v/v)

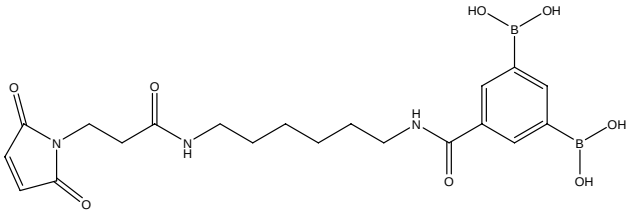
   µl PDBA-X-dithiopyridyl = $(a)(b)(c)\left(\frac{1}{d}\right)\left(\frac{1}{e}\right)10^{6}$

   where:

   a = desired molar input ratio (PDBA:protein ligand)
   b = concentration of protein ligand in mg/ml
   c = ml of protein ligand solution to be conjugated
   d = MW of protein ligand
   e = concentration of PDBA-X-dithiopyridyl, mM
10. Add the calculated volume of 100 mM PDBA-X-dithiopyridyl to the reduced protein solution and incubate at room temperature overnight.
11. *Optional*: the reaction mixture can be purified of unwanted by-products and reactants through dialysis, passing through a 500 MWCO size exclusion column, or ultracentrifugation with the appropriate filter, using a buffer of your choice.

Sulfhydryl Alkylating Reagent

Using our Versalinx® Sulfhydryl Alkylating Reagent (PDBA-X-Maleimide), PDBA can be selectively conjugated to the cysteine residues of antibody fragments or other sulfhydryl-containing proteins.



N-[6-(ß-Maleimidopropionylamino)hexyl]-3,5-bis(dihydroxyboryl)benzamide
(PDBA-X-maleimide)

Reagents and Solutions not Provided

- Sulfhydryl-containing protein
- 0.1M sodium bicarbonate, 1mM EDTA pH 7.0
- 500mM DTT in 0.1 M sodium bicarbonate, 1mM EDTA, pH 7.0
- Anhydrous N, N-Dimethylformamide (DMF) (Aldrich Part # 22,705-6 or equivalent) OR anhydrous Dimethyl Sulfoxide (DMSO) (Aldrich Part # 27,043-1 or equivalent)
- Dialysis tubing or appropriate MWCO size exclusion column or ultrafiltration tube.

Sulfhydryl Alkylating Reagent Procedure

1. Prepare a solution of the protein of interest to 10 mg/ml in 0.1 M sodium bicarbonate buffer with 1mM EDTA, pH 7.0. If the protein is already in solution, perform a buffer exchange to the above buffer, either through dialysis, using a gel filtration column, or ultrafiltration tube.
2. Measure the UV absorbance of the protein ligand solution. Using the literature value for the absorbtivity and molecular weight of the protein, calculate the concentration of the stock solution and the moles of protein to be conjugated.
3. From a freshly prepared 500 mM DTT solution, bring the protein sample to 1 mM DTT, mixing gently after DTT addition.
4. Incubate at 37˚C for 30 minutes, avoiding any further mixing to minimize reoxidation.
5. Dilute the protein sample to 0.5 mg/ml using 0.1 M sodium bicarbonate, 1 mM EDTA, pH 7.0, to decrease the interference of DTT in the conjugation reaction.
6. Bring the temperature of the diluted sample back to 37˚C.
7. Prepare a 100 mM solution of PDBA-X-Maleimide in anhydrous N,N-Dimethylformamide or anhydrous Dimethyl Sulfoxide. Vortex to dissolve.
8. Decide on the desired molar input ratio of PDBA-X-Maleimide to protein. A 10:1 molar input ratio is a good starting point. Use the following calculation to determine how many microliters of a 100 mM PDBA-X-Maleimide solution to add to the protein solution.

   The final concentration of DMF/DMSO in the conjugation reaction should be below 10%(v/v)

   μl PDBA-X-Maleimide = $(a)(b)(c)\left(\frac{1}{d}\right)\left(\frac{1}{e}\right)10^6$

   where:
   - a = desired molar input ratio (PDBA:protein)
   - b = concentration of protein in mg/mL
   - c = ml of protein solution to be conjugated
   - d = MW of protein
   - e = concentration of PDBA-X-maleimide, mM

9. Add the calculated volume of 100 mM PDBA-X-Maleimide to the protein solution and incubate at 37˚C for 30 minutes.

Optional: the reaction mixture can be purified of unwanted by-products and reactants through dialysis, passing through a 500 MWCO size exclusion column, or ultracentrifugation with the appropriate filter, using a buffer of your choice.

Determination of PDBA Modification

The actual moles of conjugated PDBA per molecule can be estimated by quantitatively comparing the absorbance at 260 nm of unmodified protein (P) versus modified protein (PDBA-P). An increase in absorbance at this wavelength is due to the PDBA addition. The molar absorbtivity of PDBA at 260 nm is 4000.

Dilute (as necessary) an aliquot of the PDBA-modified protein with an appropriate buffer. Dilute (as necessary) an aliquot of the unmodified protein with the same buffer. Measure A280 and A260 of both solutions. Use the following equations to determine the degree of modification. "DF" refers to the dilution factor used to determine absorption, and "a" is the absorbtivity of the protein at 280 nm.

$$\left[\text{PDBA - P, } {}^{\text{mg}}\!/_{\text{ml}}\right] = A_{280}^{\text{PDBA}-\text{P}} \Big/ a_{280}^{\text{P}}$$

$$\left[\text{PDBA - P, M}\right] = \frac{\left[\text{PDBA - P, } {}^{\text{mg}}\!/_{\text{ml}}\right]}{\text{MW}^{\text{P}}}$$

$$A_{260}^{\text{PDBA}} = (\text{DF})\left(A_{260}^{\text{PDBA-P}}\right) - \left((\text{DF})\left(A_{260}^{\text{PDBA-P}}\right)\left(A_{260}^{\text{P}} \Big/ A_{280}^{\text{P}}\right)\right)$$

$$\left[\text{PDBA, M}\right] = A_{260}^{\text{PDBA}} \Big/ 4000$$

$$\text{Incorporation ratio of PDBA} = \frac{\left[\text{PDBA, M}\right]}{\left[\text{PDBA - P, M}\right]}$$

Safety: Refer to applicable MSDS's

References

1. Hermanson, G.T.,(1996) Bioconjugate Techniques, *Academic Press, San Diego*: 139-140.
2. Stolowitz, M., Ahlem, C., Hughes, K., Kaiser, R., Kesicki, E., Li, G., Lund, K., Torkelson, S., and Wiley, J. (2001) Phenylboronic Acid-Salicylhydroxamic Acid Bioconjugates I: A Novel Boronic Acid Complex for Protein Immobilization. *Bioconjugate Chem* **12:** 229-239 .
3. Wiley, J., Hughes, K., Kaiser, R., Kesicki, E., Lund, K., and Stolowitz, M.,(2001) Phenylboronic Acid-Salicylhydroxamic Acid Bioconjugates II: Polyvalent Immobilization of protein ligands for Affinity Chromatography. *Bioconjugate Chem* **12:** 240-250.

For Research Use Only; Not for use in diagnostic procedures.

Ordering Information
Versalinx® Experimenter's Kit 50602

50602 Kit Contents

Protein Modifying Reagents

- 50597 Versalinx® Amine Modifying Reagent (FW 500.11) 5mg
- 50598 Versalinx® Carbohydrate Modifying Reagent (FW 336.80) 5mg
- 50599 Versalinx® Disulfide Forming Reagent (FW 336.80) 5 mg
- 50560 Versalinx® Sulfhydryl Alkylating Reagent (FW 459.07) 5mg

Packaged separately

- 50592 Versalinx® SHA-Agarose Chromatography Media, 5ml of a 50% v/v slurry

Storage

All Versalinx Protein Modifying Reagents should be stored at -20°C.

Versalinx® SHA-Agarose Chromatography Media should be stored at 4°C.

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