FT-BL1501

ControlledAmine™ Conjugation kit

Description

Complete kits for the conjugation and oriented immobilization of aminated biomolecules – with stable activation!.

ControlledAmine™ Conjugation kit1 . BL1501, 1 kit . BL150A, 1 kit*

contains: SANH Reagent (25 mg); MW: 290
SFB Reagent (100 mg); MW: 314
pNBA Reagent (p-Nitrobenzaldehyde,100 mg); MW: 151
2HP Reagent (2-HydrazinoPyridine Reagent, 100 mg); MW: 182
2SBA Reagent (2-SulfoBenzAldehyde Reagent, 50 mg); MW: 208

ControlledAmine™ Conjugation kit2 . BL1521, 1 kit . BL152A, 1 kit*

contains: SHTH Reagent (25 mg) MW: 313
SFB Reagent (100 mg); MW: 314
pNBAReagent (p-NitroBenzAldehyde,100 mg); MW: 151
2HP Reagent (2-HydrazinoPyridine Reagent, 100 mg); MW: 182
2SBA Reagent (2-SulfoBenzAldehyde Reagent, 50 mg); MW: 208

* kits BL150A and BL152A include additionally DMF (1ml), 10x Modification buffer (1.5ml), 10 Conjugation Buffer (1.5ml), 4 Diafiltration units (5KDa MWCO)

Storage: Room temperature (≤ -18°C)
(prolonged storage: keep cool and dry and under argon)

Introduction

Here is a great way to get started with easy and performing bioconjugation chemistry!

Simply modify each molecule and mix to yield the desired compound!

You get oriented and active conjugates with high yield, which will allow you forgot conventional chemistry with glutaraldehyde or SMCC. The kits include protocols and full reagents for conjugation of biomolecules, and additional colorimetric reagents for quantitation of aldehyde and hydrazide incorporation.

Benefits of the chemistry: *increased ease of use, reproducibility and efficiency in bioconjugations*

- Biomolecules can be activated independently
- Better control of coupling ratio (quantitate modifications before coupling)
- Activated biomolecules are stable for months
- No reduction or deprotection step is required.
- Excellent yields, in organic solvents, as well in aqueous buffered solutions
- Highly selective for heteroconjugation (oriented and controlled conjugation: no homoconjugate)
- Very stable Conjugates that retain inherent biological activity of its components

Applications: *flexible technique for a variety of molecules*
-suits for proteins and any amine containing or derivatized molecules and supports: have been successfully coupled.
proteins, peptides **carbohydrates, oxidized** **fluorophores,**
oligonucleotides, cDNA, **glycoproteins,** **beads, glass, silica**...
-suitable for **surface modification** and conjugation (immobilization)
-suitable for **solid phase synthesis** (oligonucleotides, peptides)

Directions for use

The ControlledAmine™ conjugation kits include protocols, reagents for aldehyde and hydrazide incorporation (SANH/ SHTH, SFB), for conjugation of biomolecules and quenching (2-SulfoBenzaldehyde), and additional colorimetric reagents for quantitation of aldehyde and hydrazide incorporation (4NBA, 2HAP). All are powder form, and to store at room temperature desiccated. Kits BL150A and BL152A include also buffers and diafilter device.

The reagents are for research use only. Not for use in diagnostics

Procedure time	6-8hours	
Min.amount of material	Protein: 25µg Oligonucleotide; 10µg Chromophore: 4µg	
Number of reactions:	10	
Reaction Yield and stability:	*SANH modification:*	˜50% stable 2 months at –20°C
	SHTH modification:	95%, stable 2 months at –20°C
	SFB modification:	95%, stable 2 months at –20°C
	Conjugation:	100%, stable 12 months at –20°C

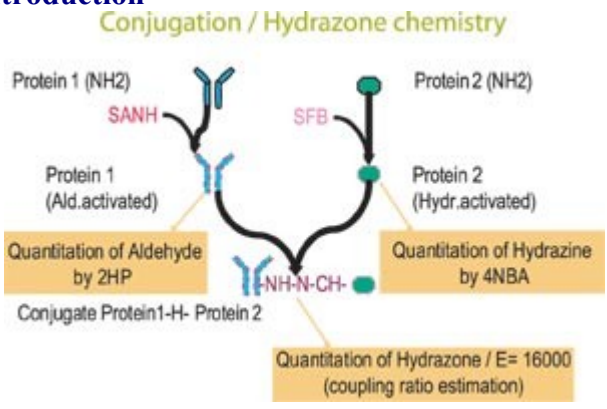
Prior to use, allow reaching at room temperature before opening reagents.
After opening, SANH, SHTH and 2SBA dissolved in anhydrous DMF are stable for 1 month at room temperature. The 2HP and 4-NBA dissolved in Conjugation Buffer are stable for 1 month at +4°C. The 4-NBA 2HP and 2-SBA reagents must be protected from light.

Not provided (provided in kits BL150A/BL152A)
-Solvents (**anhydrous DMF**)
-Modification Buffer: 100mM phosphate, 150mM sodium chloride pH7.2-7.4. Standard PBS buffer containing 10mM phosphate is not recommended for protein modification due to poor buffering capacity.
-Conjugation Buffer:
10mM phosphate, 150mM sodium chloride, pH6.0 is recommended for antibodies and other proteins.
Many buffers can be used for conjugation, such as 100% sodium/citrate, 150mM NaCl pH6.0, or 100mM sodium acetate pH5.0 for other biomolecules.
-Desalting: dialfilters are provided in kit "A". Or choose gel filtration such as UP84874 (for <3ml volumes), diafilters such as UptiSpin Concentrator BB9300, or dialysis membranes such as CelluSep tubings (for large volumes). Please refer to supplier to check suitability for your samples.

Protocol 1: Protein to Protein conjugation -> Introduction

The conjugation includes 2 simple steps, using the Hydralink™ technology:

1-modification of amines from biomolecule1 in hydrazine (-NH-NH2) or hydrazide by reaction with SANH or SHTH, and modification of amines from biomolecule2 in aldehydes by reaction with SFB.
2-conjugation of activated molecules to form a stable hydrazone bond (-NH-NH-)
3-desalting



The Hydrazone chemistry is
simple, flexible, selective and reproducible !

The following protocol works for SANH and SHTH kits. It is based on standard conditions (i.e. 10-15 equivalents of modifier). For more information, please refer to section ' Technical and Scientific Information'.
Please as Uptima if you need help for calculations, especially for different types of biomolecules.

1a: –> SANTH or SHTH activation

1.Prepare a solution of SANH or SHTH by dissolving 2mg of reagent (or required equivalents) in 100µl DMF.
Note: pay attention the to a different molecular weight, amounts of SANH and SHTH required for conjugation may differ slightly upon conjugation.

2.Add the required volume of SANH or SHTH solution to the biomolecule: standard conditions are 10-15 equivalents of modifier for to a 2mg/ml protein solution. For more information, please see below.
Note: We recommend using at least 1mg of oligonucleotide of 0.1mg of protein per reaction. Keep< 10% DMF if the biomolecule was sensitive.

3.Incubate at room temperature for a minimum of 2 hours

4.Collect activated biomolecule using a gelfiltration column, diafiltration or dialysis.
Please refer to desalting tool supplier for operating procedure.

5.Calculte the biomolecule concentration using a colorimetric method such as BC Assay (UP40840) or Bradford Assay (UPF4600) or any other suitable assay.

6.Quantitate hydrazine incorporation.

6.1.Prepare a 0.5M p-NBA solution: Dissolve pNBA in DMF, and add the required volume to Conjugation Buffer. The solution is stable for 1 month at+4°C when protected from light.

6.2.Add an aliquot of the Hydrazine-modified protein solution to the pNBA solution and incubate at +37°C for 1 hour, or at room temperature for 1 hours. Determine the absorbance at 390nm against a blank (equal aliquot of buffer + pNBA solution).

6.3.The **hydrazine/protein MSR = Abs_{380nm} / 22 000 / (protein concentration in mol/L)**
Note: if the number of reactive 2-hydrazinopyridine moieties on the biomolecule is a problem for quantitation, because of biomolecule precipitations for instance, use a solution of SBF 0.5mM in Conjugation buffer instead of pNBA (follow the same protocol).

1b: –> SFB activation

1.Prepare a solution of SFB by dissolving 2mg of reagent in 10µl DMF.

2.Add the required volume of SFB solution to the biomolecule: standard conditions are 10-15 equivalents of modifier for to a 2mg/ml protein solution. For more information, please see below.
Note: We recommend using at least 1mg of oligonucleotide of 0.1mg of protein per reaction. Keep< 10% DMF if the biomolecule was sensitive.

3.Incubate at room temperature for a minimum of 2 hours.

4.Collect activated biomolecule using a gelfiltration column, diafiltration or dialysis.
Please refer to desalting tool supplier for operating procedure.

5.Calculate the biomolecule concentration using a colorimetric method such as BC Assay (UP40840) or Bradford Assay (UPF4600) or any other suitable assay.

6.Quantitate aldehyde incorporation.

6.1.Prepare a 0.5M 2HP solution: Dissolve 2HP in DMF, and add the required volume to Conjugation Buffer. The solution is stable for 1 month at +4°C when protected from light.

6.2.Add an aliquot of the benzaldehyde-modified protein solution to the 2HP solution and incubate at +37°C for 1 hour, or at room temperature for 2 hours. Determine the absorbance at 390nm against a blank (equal aliquot of buffer + 2HP solution).

6.3.The **aldehyde /protein MSR = Abs_{350nm} / 18 000 / (protein concentration in mol/L)**

1c: –> Conjugation

1.Mix the activated the hydrazine-protein1 (SANH or SHTH-activated) and benzaldehyde-protein2 (SFB-activated)
-for proteins: at a 1:1 molar ratio,
-for oligonucleotides: at a 1:1.5 molar ratio
-please refer to section 'Technical and Scientific Information' for specific applications (high MW conjugate preparation)

2.Incubate for 2-16 hours at room temperature in Conjugation buffer.

3.The extent of conjugation can be determined on an aliquot of the reaction mixture by SDS-PAGE analysis of a protein-protein conjugate, or by PAGE analysis for protein-oligonucleotide conjugate.

4.The excess hydrazinopyridine moieties on the biomolecules, still reactive after conjugation, can be quenched by using a solution of 2-SBA 0.5mM in Conjugation Buffer for 2 hours.

5.Desalt the conjugate. The conjugate can be isolated by standard size exclusion chromatography (gelfiltration columns UP84874), diafiltration (provided unit in kits 'A'; or UptiSpin), or dialysis (CelluSep). For purer conjugates, we recommend FPLC to isolate protein-protein, and size exclusion for protein-oligonucleotide conjugates.

Technical and Scientific Information

- Principle

The conjugation includes 2 simple steps, using the Hydralink™ technology: see directions for use.
Hydralink™ is covered by US Patents 5,206,370, 5,420,285, 5,753,520, and 5,769,778, and EU Patent 0.384,769.

- Advantages over alternative strategies for oriented coupling chemistries

Maleimide/succinimidyl chemistry (SMCC type) has become a standard for protein-to-protein oriented conjugation.
Despite very good performances in coupling, the technique suffers of several limitations. Because it needs free sulfhydryl presence in one molecule to be coupled, it require, at least for molecules devoid of Cys, a genetic engineering for introduction of Cys residues, or a biochemical conversion of other groups (amines) into sulfhydryls. The biochemical reduction of already present disulfide bridges will lead to non-native state coupled molecules. Because of sulfhydryls susceptibility to oxidation by air, a reduction by DTT before conjugation is usually performed, that requires an additional desalting step. Succinimidyl, but also maleimide, should react in timed delay because of hydrolysis competition, hampering the final yield. As Cys residues are often involved in the biological activity, the maleimide strategy leads then to unstable or inactive conjugates. Finally, intramolecular crosslinking may occur (NH2 and SH bearing molecules), crosslinking is quite polymorphic, and coupling ratio are not easy to control*.

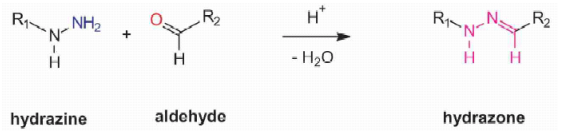
Avidin/Biotin has also been proposed to create oriented conjugations, with several advantages: the binding is rapid and stable. However, the use of avidin, a rather big protein, limits it' use: avidin conjugates do no permeate cell membranes, induce immunogenic response, and give noticeable background in certain detection systems because of hydrophobic&ionic interactions. This has lead to prefer using streptavidin, a bacterial protein, with is more neutral. However, affinity for biotin is slightly lower, and a new drawback is undesired binding to certain adhesion molecules by affinity of its RGD sequence. Also, a chemical neutralized avidin has been developed. Whatever, Avidin/Biotin systems are useful in signal amplification systems, rather than for bioconjugation of probes.

Glutaraldehyde chemistry has been extensively used, but is not selective. Even with a 2 step method, a large proportion of formed conjugates are dimers of parent molecules, and intramolecular crosslinking is important.

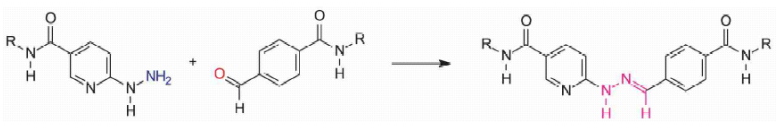
Hydrazone technology provides solutions to each of these points:
-Proteins or other molecules that lack Cys residues or which Cys residues are involved in II structure or function, can be easily coupled.
-Biochemical modifications are distinct in the 2 biomolecules, and avoid intramolecular crosslinking.
-Biochemical modifications are stable, hence can be better quantified to adjust concentration ratio for the coupling step. Modified proteins can even be stored and different couplings can be performed with the same batch for greater flexibility with excellent reproducibility.
-Other modifying agents allow coupling schemes to thiols (with MHPH), Silanols (with Hydrazine silane)

- Chemistry

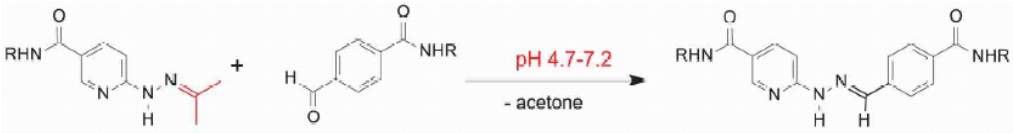
The ControlledAmine™ is based on Hydralink™ technology, a hydrazone chemistry:
The 2 molecules to be coupled are first modified to introduce hydrazine and aldehyde groups, using reactive agents SANH and SFB respectively. The modification occurs on primary amino groups (-NH2) such a thus present on the side chains of lysine residues and N-termini of proteins, or any primary amine present in other molecules (aminoallyl nucleotides, aminated lipid...).
Then, the modified biomolecules are simply mixed together to yields the hydrazone-mediated conjugate:



The reaction is acid catalyzed, with an optimal pH at 4.7 and a range up to pH8.0, albeit with slower kinetics. No reducing agents are required (unlike NHS/Maleimide or glutaraldehyde chemistry, see below advantages), thereby decreasing chances of inactivating the biomolecules. Therefore, no reduction of native disulfide bonds occurs in proteins, and bio-activity is retained.
Specifically, the HydraLink™® chemistry in SANH/SFB kit is based on the reaction of 2-hydrazinopyridyl moiety with a benzaldehyde moiety, yielding a stable bis-aromatic hydrazone:



The incorporation of benzaldehyde moieties on proteins is performed with SFB reagent.
The incorporation of hydrazine moiety on the other protein is performed as desired by SANH or SHTH reagents, respectively as hydrazinopyridine and a 4-hydrazidoteraphtalate. The hydrazine group in SANH is protected as its acetone hydrazone. This alkyl hydrazone is not stable in mild acid and rapidly exchanges with an aromatic aldehyde during conjugation, yielding a stable bi-aromatic hydrazone. No separate deprotection step is required.

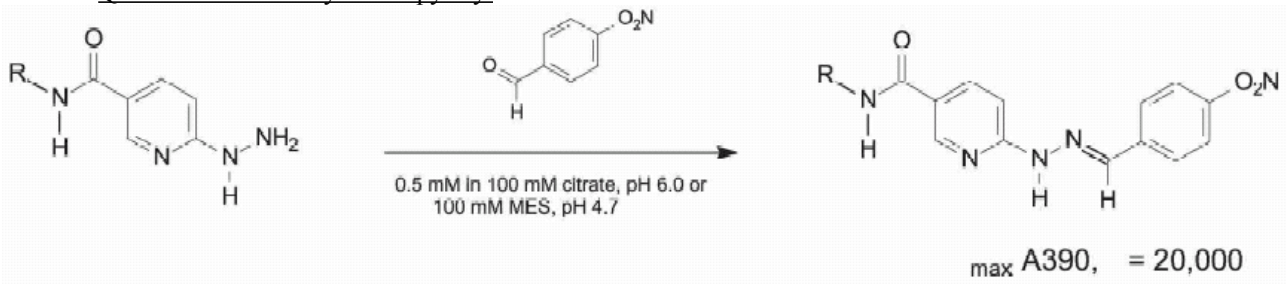


The SHTH converts amine groups to aromatic hydrazide groups that can form stable bis-aryl hydrazide hydrazones. The SHTH chemistry yields an even more stable modified biomolecule that the SANH chemistry.

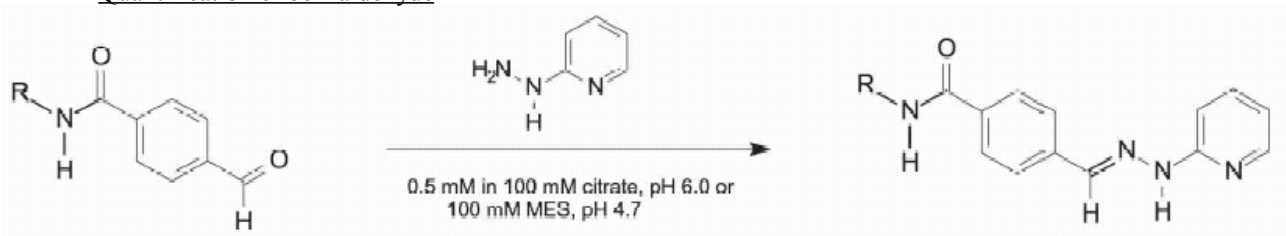
- Advantages

- **No reduction reagents** are required to stabilize the bond, unlike :
  -in NHS/Maleimide that needs reduction of disulfides i.e. with DTT
  -in glutaraldehyde chemistry that needs reduction of Shiff's base i.e. with Na Cyanoborohydride.
- **Very high selectivity**; do not lead to inter-subunit crosslinking, or homo-crosslinking, unlike to NHS/Maleimide strategy (i.e. SCMCC).
- **Flexible method**: Reagents are available to incorporate aldehyde moieties on oligonulcleotides and hydrazine moieties on peptides during solid phase synthesis.
- **The level of incorporation** of 2-hydrazinopyridyl and benzaldehyde can be quantified using spectrometric methods after protein modifications, and before conjugation.

- Quantification of 2-hydrazinopyridyl



- Quantification of benzaldehyde



- Degree of conjugation / Molar Substitution Ration (MSR)

The yield of conjugation is usually excellent, above 90% (much superior to NHS/Maleimide method). It depends essentially on the yield of incorporation of the modification moieties (a function of the modifier and biomolecule concentrations), as the conjugation reaction itself has a 100% yield. We recommend initially adding 10-15 equivalents of modifier to a 2mg/ml protein solution. We recommend also optimizing the modification conditions for each new protein, as the over-modification may result in precipitation, loss a biological activity.

The degree of substitution by Benzaldehyde was determined on goat IgGs with 0. 5. 10, 20, 30, 40, 50 equivalents of SFB. The UV absorbance of IgG between 300 and 350 nm is not significantly changed by SFB modification, but a slight of the spectra (OD versus wavelength) shift to the left is observed. Finally, the MSR was found as follows:

Equivalents of SFB/IgG	mg/ml	MSR
0	0.325	0
5	0.414	1.23
10	0.321	4.12
20	0.301	5.7
30	0.283	8.61
40	0.272	11.6
50	0.260	13.7

- Biomolecule preparation

The biomolecules to be activated with SANH/SHTH and SFB must be in a buffer completely free of amine groups. Buffers containing Tris or Glycine cannot be used because of interference. We recommend adjusting the biomolecule solution by using a 1x or 10x solution of Modification Buffer by dialysis or dilution steps.

TROUBLE SHOOTING

Problem	Possible cause	Recommandation
Poor modification	initial biomolecule concentration too low	concentrate biomolecule 2-4times, use a 2-5mg/ml protein solution for example
	insufficient equivalent of modification reagent added	add more modification reagent equivalent for modification reaction, up to 50 equivalents can be used
	amine contaminant e.g. Tris or glycine present in starting solution	exchange the buffer by diafiltration, dialysis or desalting
Quantitation assay readings out of range	precipitation of the modified protein on treatment with quantitation reagent leads to spurious reading	for hydrazine modified proteins use 2-SB for quantitation
Precipitation of protein on modification	over-modification of protein	modify with less equivalents modification reagent
Protein/Protein conjugate has too high molecular weight	due to high modification levels on each protein a large MW product is formed	reduce the modification levels of (one) starting(s) protein(s)

Other Information

*** Related products: other Hydrazone chemistry reagents**

Components of kits #BL1501 and BL152, SANH & MHPH, SFB & MTFB reagents are available separately. Other reagents for hydrazone chemistry are available, i.e. silane derivatives, linkers and building blocks for organic synthesis. See related product in document FT-BL9270, or please inquire.

*** Related products: desalting tools**

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