Application Note

Thermo Scientific Nunc Immobilizer Amino Surface

Protocol for Coupling Proteins

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Key Words

Thermo Scientific™ Nunc™ Immobilizer™ Amino surface, peptide coupling, protein coupling, immuno-diagnostic assay, ELISA, microtiter plate.

Goal

The goal of this Application Note is to describe the coupling protocol for proteins when using the Amino Immobilizer. In addition to provide a guideline if coupling thiols alone as well as coupling amine and thiol groups.

The Nunc Immobilizer reagent range is manufactured using a patented photo-chemical method [1] for covalent coupling of ligands to polymer materials.

The photo-coupling introduces an ethylene glycol spacer and a stable electrophilic group that reacts with nucleophiles such as free amines, thiols or hydroxy groups. The spacer design and the density of electrophilic groups on this surface are optimized for peptide and protein based immuno-diagnostic assays.

Background

The proprietary Nunc Immobilizer reagent (Fig. 1) incorporates an electrophilic functional group that will react with any good nucleophile.

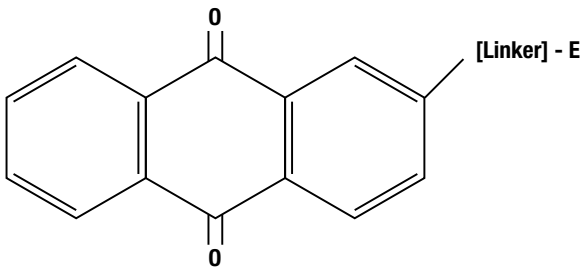

Fig. 1

Proprietary AQ Nunc Immobilizer reagent. The electrophilic group, E, is separated from the photo-reactive anthraquinone via an ethylene glycol linker.

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In the case of proteins, this essentially limits reaction to primary amine and thiol groups (Fig. 2). Other potential nucleophilic groups on proteins, such as tyrosine hydroxyl and histidine imidazole groups, are either not nucleophilic at the pHs where most proteins are stable, or are such weak nucleophiles that they are competed out by both the overabundance and higher reactivity of the highly nucleophilic amine and thiol groups. Furthermore, reactivity of the amine and thiol groups is a function of pK_a and can be modulated by pH. Thus, amines are poor nucleophiles when they are protonated and are expected to react less vigorously at pHs below the pK_a of the ε-amine group, whereas the thiol group of cysteine reacts more vigorously in the thiolate form found above the pK_a of the thiol group.

Therefore, it may be possible to limit reaction to thiol groups by running reactions closer to neutral pH where the thiolate anion will be the most reactive species. These considerations must be weighed with care, as the pK_as of ionizable groups in proteins are often very different from the pK_as of the respective amino acid side chains in solution, and therefore may not reflect the reactivity of the potential nucleo-philicity of these groups in proteins.

Suggested Guidelines and Protocol

The chemistry of proteins is very diverse, and it is therefore difficult to develop a generalized protocol for coupling with the Nunc Immobilizer reagent that works well for all proteins. A certain amount of optimizing may be necessary to obtain the best results. Since the target nucleophiles are either primary amines or thiols, the Proteomics group at Exiqon employed two different conditions for the covalent coupling reactions. One of these conditions will usually provide at least acceptable results, but users are encouraged to take into consideration the individual properties of the proteins with which they are working and develop their own set of conditions, if need be.

- Proteins are coupled to the Nunc Immobilizer Amino reagent using two different buffer systems:

  -Phosphate buffered saline (PBS; 10 mm Na phosphate buffer, pH 7.5, 150 mm NaCl)

  -100 mm Na carbonate, pH 9.6

PBS will favor coupling thiols alone, whereas carbonate will facilitate reaction with both amine and thiol functions.

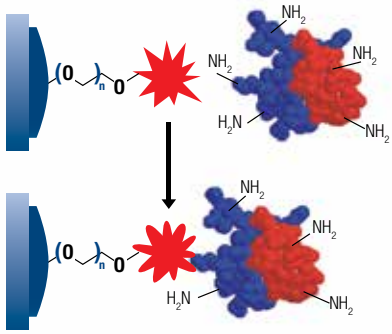

Fig. 2.

Coupling of protein to surface bound AQ Immobilizer reagent.

- When coupling proteins to surfaces coated with the Nunc Immobilizer reagent, it is suggested that a dilution series of the protein of interest be run in the two buffers above.
  A suitable protein concentration at which to start is around 100 µg/mL. Coupling times vary and should be determined empirically, but a good starting place is a one hour incubation at ambient temperature. Be aware that both temperature and protein concentrations affect the reaction rate. Furthermore, amine buffers and other nucleophiles should not be present in the protein solutions used for immobilization.

- After coupling to the surface, remaining Nunc Immobilizer electrophilic groups are quenched by reaction with 10 mm ethanolamine in 100 mm Na carbonate, pH 9.6 buffer for one hour at ambient temperature. This eliminates the possibility of the surface reacting at a later point in time with other nucleophiles and also introduces a hydroxyl functional group which makes the surface more hydrophilic and less prone to non-specific absorbtion. The surface can now be used for immuno-assay purposes without the need for a non-relevant blocking protein to be present in assay buffers.

Additional information

More information concerning immobilization of proteins on the Nunc Immobilizer Amino surface can be found on www.thermoscientific.com.

References

1. Jensen SP, Rasmussen SE, Jakobsen MH. Photochemical Coupling of Peptides to Polystyrene MicroWell Plates. Innovations and Perspectives in Solid Phase Synthesis and Combinatorial Chemical Libraries (1996) 419-422.

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