Application Note

Covalent Binding

Thermo Scientific Nunc Covalink NH

Using simple, well-understood chemistry this process allows the user to covalently immobilise molecules to the NH functional groups grafted to the polystyrene.

In order to give an overall impression of the options offered by the use of Thermo Scientific Nunc CovaLink, we present a number of examples of how the surface can be used.

We hope that these will act as inspiration for you to develop your own assay applications.

The Concept

Nunc™ CovaLink™ NH was developed to bind compounds covalently to a plastic surface.

The product is a polystyrene surface to which secondary amino groups have been grafted by means of a spacer arm. The spacer arms are approximately 2 nm long and are grafted to the surface at a density of 10^{14}/cm² (Fig. 1).

This offers a number of advantages:

Highly specific binding

Secondary amino groups have been chosen for this application as they are stable groups of moderate activity. This ensures that coupling of the specifically activated reagents occurs while non-specific coupling is kept to a minimum.

Easily accessible reactive sites

Placing the secondary amino group at the end of a spacer arm allows unhindered access to molecules in the liquid phase. The distance of 2 nm has been found to be optimal for most applications.

Orientation of immobilized component

Unlike simple adsorption the orientation of the immobilized molecules can be influenced by controlling the defined reaction conditions. In this way exposure of areas of biological activity to reagents in the liquid phase of the assay may be ensured (Fig. 2).

High binding capacity

The surface density of 10^{14}/cm² is sufficient to allow saturation by most molecules.

Hapten binding possible

Use of covalent binding allows stable immobilization of small haptenic molecules which bind weakly or not at all by physical adsorption.

Prior to the binding of the molecule to be immobilized there is an activation step. Activation can be achieved a number of ways which are illustrated by examples in the next section.

Note

As N-hydroxysuccinimide (NHS) is poorly soluble in water. Sulfo-NHS has been used in the examples which follow. In the interest of simplicity, in the reaction schemes, NHS is used.

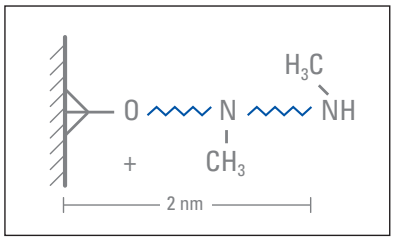

Fig. 1.
Schematic chemical and physical configuration of the CovaLink NH surface. The NH groups are distanced from the polystyrene surface by approx. 2 nm long, chemically defined, spacer arms covalently anchored to the surface. The density of grafted complexes is approx. 10^{14} cm^{-2}.

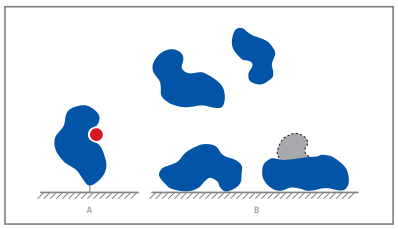

Fig. 2.
Schematic illustration of the advantage of orientated covalent immobilisation in contrast to physical adsorption of molecules.

A: Covalent coupling, disposed on a spacer arm, may occur via a group away from the molecule's active site (crescent groove) to ensure an orientation of the molecule which makes its active site accessible for the target molecules (●) added in the liquid phase.

B: Physical adsorption involves the risk of hiding the active site against the surface (left), or destruction of the active site due to distortion of the molecule (right) by adsorption.

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Application

Coupling NHS activated compounds

One application of CovaLink NH is the coupling of molecules that have been activated by esterification with N-hydroxysuccinimide (NHS).

Such active esters link immediately to the surface amino group as shown in the reaction scheme in Fig. 3 using NHS-biotin.

Preparation of reagents and buffers

Materials

- Solid Phase: CovaLink NH, Thermo Scientific Nunc MaxiSorp, plate without surface treatment
- N-hydroxysuccinimide-biotin (NHS-biotin)
- Dimethyl sulfoxide (DMSO)
- Bovine serum albumin (BSA)
- Tween 20
- Triton X-100
- Avidin
- Avidin horseradish peroxidase conjugate (Avidin-HRP)
- Ortho-phenylenediamine dihydrochloride (OPD)
- Hydrogen Peroxide (H2 02), 30%
- Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC)
- Disuccinimidyl suberate (DSS)
- Alpha-foetoprotein (AFP)
- Casein
- Rabbit anti-AFP antibody horse radish peroxidase conjugate (Ra AFP-HRP)

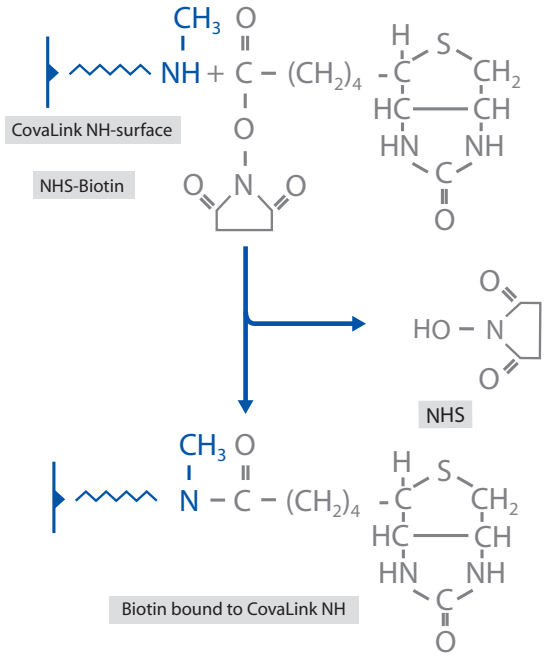


Fig. 3.
Reaction scheme for immobilisation of NHS-biotin to the CovaLink NH surface through NHS splitting off.

NHS-biotin stock solution

NHS-biotin 100mg
DMSO ..... ad 10mL
Store at 4°C

NHS-biotin solution

NHS-biotin stock solution. 1250µL
PBS ..... ad 100mL
Note: Use fresh solution

AFP solution

AFP in carbonate buffer ..2.5µg/mL

Conjugate Solution

Ra AFP-HRP (final concentration)
.....2.5µg/mL
in PBS + 0.05% Tween 20

DSS solution

DSS 12.5mg
DMSO 50mL
Carbonate buffer ..... ad 100mL
Note: Dissolve DSS in DMSO before dilution in carbonate buffer. Use only fresh solution.

Avidin mix

Avidin 400µg
Avidin-HRP 13µg
Cova Buffer ..... ad 100mL
Note: Use fresh solution

Substrate solution

H_2O_2 (30%) 50µL
OPD 60mg
Citrate-phospate buffer.....
..... add 100mL
Note: Use fresh solution and keep dark.

Phosphate Buffered Saline (PBS)

0.15M, pH 7.2
NaCl 8.0g
KCl 0.20g
$Na_2HPO_4 \cdot 2H_2 0$ 1.15g
KH_2PO_4 ..... 0.20g
Distilled water ..... ad 1000mL
Adjust to pH 7.2 with HCl/NaOH

Washing buffer

NaCl 12.2g
Triton X-100 ..... 0.5mL
PBS ..... ad 1000mL
Adjust to pH 7.2 with HCl/NaOH

Blocking buffer

Casein 5g
PBS ..... ad 1000mL

CovaBuffer

NaCl 116.9g
$MgSO_4 \cdot 7H_20$ 10.0g
Tween 20 ..... 0.5mL
PBS ..... ad 1000mL

Carbonate buffer pH 9.6

Na_2CO_3 ..... 1.59g
$NaHCO_3$ 2.93g
Distilled water ..... ad 1000mL
Adjust to pH 9.6 with HCl/NaOH

Citrate-Phosphate buffer

0.1M, pH 5.0
Citric Acid, $C_6H_8O_7 \cdot H_20$ 7.30g
$Na_2HPO_4 \cdot 2H_20$ 11.86g
Distilled water ..... ad 1000mL
Adjust to pH 5.0 with HCl/NaOH

Example 1

This example has two purposes:

1. To demonstrate that in the absence of NHS no binding of biotin is observed.
2. To compare the binding of biotin in CovaLink and untreated wells.

A. Incubation

- Set up two CovaLink plates (A with and B without NHS) and C, a plate without surface treatment.
- To plates A, B and C, add 100µL PBS to all wells except those in column 2.
- Add 200µL NHS-biotin to all wells in column 2. Make a serial dilution by transferring 100µL from the wells in column 2 to those of column 3 and mix. Repeat the process in all subsequent columns. After mixing, discard 100µL from the wells in column 12 (Fig. 4).
- To plate B, add 100µL PBS to all wells.
- Cover the plates.
- Incubate overnight at room temperature.

B. Wash

- Empty the wells and wash three times with CovaBuffer.

C. Conjugate Incubation

- Empty the wells, add 100µL Avidin mix to each well, cover the wells and incubate for two hours at room temperature.

D. Wash

- Empty the wells and wash three times with washing buffer.

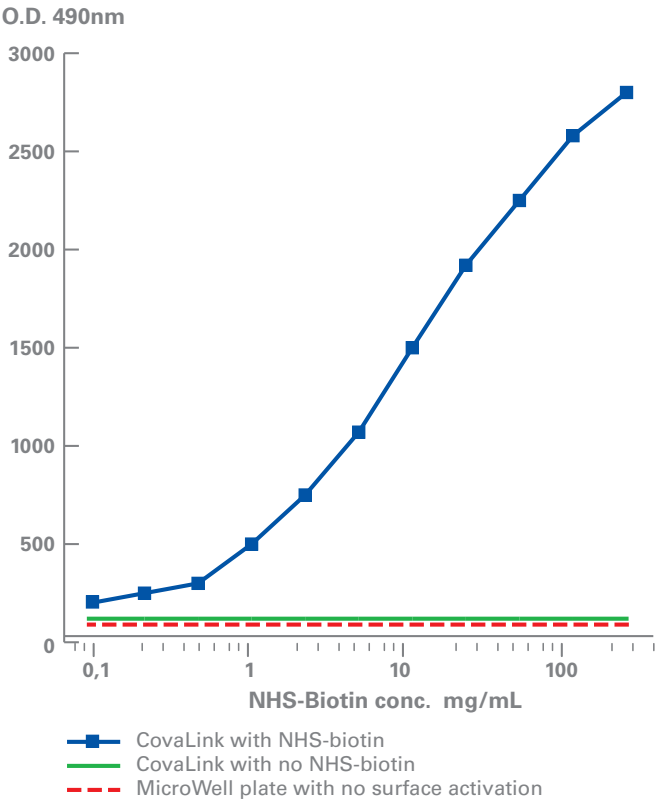


Fig. 5.
Biotin binds to the CovaLink NH surface in correlation with the amount of NHS-biotin added.

E. Substrate Reaction

- Empty the wells.
- Add substrate solution, 100µL/well.
- Wait for color development and stop the reaction by adding 1M H_2SO_4, 100µL/well.
- Read O.D. of wells at 490nm.

F. Results

- In the absence of NHS no passive adsorption of biotin is observed (Fig. 4).
- The result (Fig. 5) show a clear correlation between the concentration of NHS-biotin added to the well and the amount of biotin bound to the CovaLink NH surface.
- On the plate without secondary amino groups grafted to the surface, no biotin was found.

G. Conclusion

- Biotin has been covalently bound to the amino groups on the CovaLink NH surface.
- No biotin or enzyme conjugate was passively adsorbed.

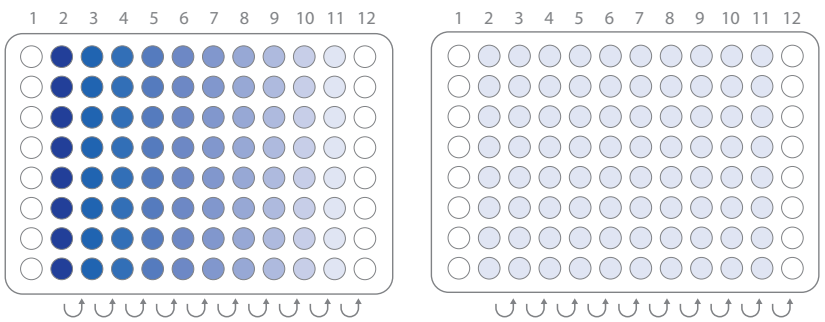


Fig. 4.
The plate on the left shows the effect of the presence of NHS. The dilution of biotin is clear in comparison with the control plate on the right.

Example 2

The purpose of this experiment was to demonstrate that secondary amino groups can be blocked using NHS-activated compound sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), added to the wells prior to incubation with NHS-biotin.

A. Incubation

- Three CovaLink plates were used. A) with SMCC and NHS-biotin, B) without SMCC but with NHS-biotin and C) without SMCC and NHS-biotin.
- For plate A), a 1:2 serial dilution of NHS activated compound (SMCC dissolved in DMSO and diluted in PBS) was prepared in the wells to generate the concentrations shown in Fig. 6. In plates B) and C) make a dilution without SMCC (buffer unly). Cover the plates.
- Incubate overnight at room temperature (RT).

B. Wash

- Wash the plates three times with CovaBuffer.

C. Incubation

- Empty the wells. To plate A) and B), add NHS-biotin (excess). To C), add buffer only.

D. Visualisation

- Follow the instructions in example 1 steps D and E

E. Results

- The blocking effect of increasing concentrations of SMCC can be seen as a decrease in bound biotin in plate A (Fig. 6).

F. Conclusion

- The NHS-activated compound SMCC binds to the CovaLink NH surface, blocking the secondary amino groups and preventing further binding of NHS-biotin.

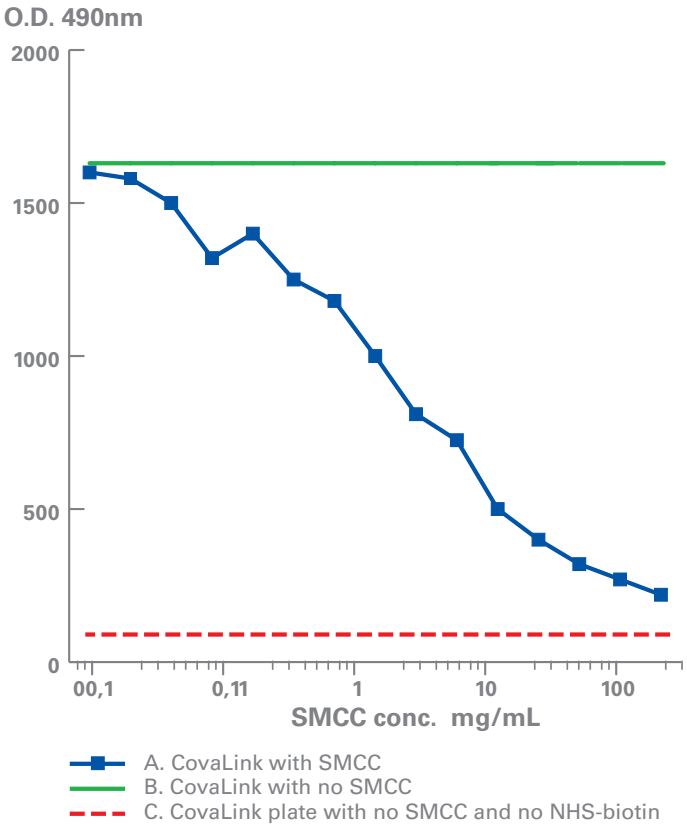
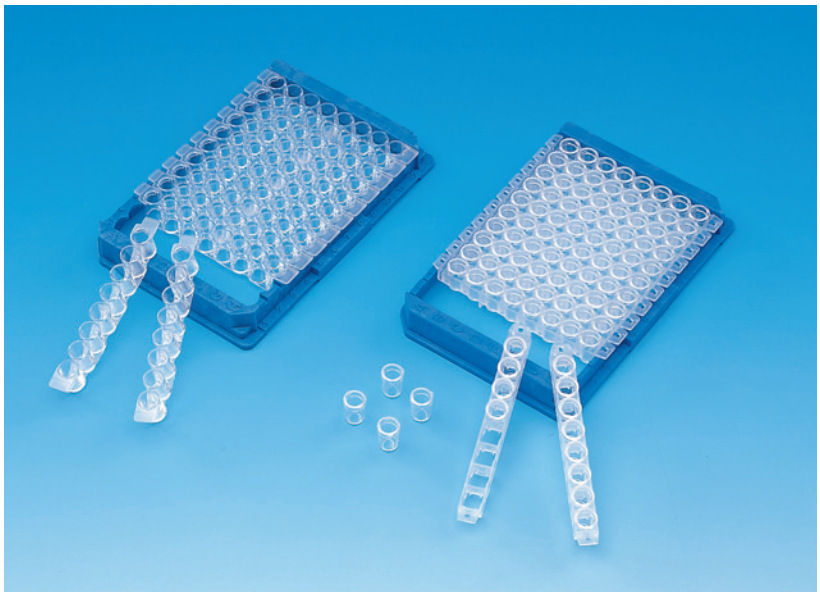


Fig. 6.
The binding of biotin to the CovaLink NH surface was reduced by blocking the secondary amino groups with increasing amounts of SMCC.



CovaLink NH Modules, 96 wells per frame. Extenal dimensions 128 x 86 mm. Configuration, F8 and C8 Break Apart.

Example 3

The purpose of this experiment was to demonstrate that CovaLink NH is not only suitable for immobilization of small molecules, e.g. biotin, but also for large molecules, e.g. AFP, using bifunctional linkers.

As an example of a large molecule, we have chosen the protein antigen alpha foetoprotein (AFP) (MW 70.000).

Physical adsorption of AFP to MaxiSorp™ and Thermo Scientific Nunc PolySorp was compared with coupling of AFP to CovaLink NH using the homobifunctional linker, disuccinimidyl suberate (DSS). This linker has an active NHS ester group at both ends.

The DSS coupling method does not allow for orientation control, because the coupling may be established through any available amino group in the molecule. Since one single covalent bond is sufficient for stable immobilization, it could be anticipated that a much larger part of the immobilized molecule would be available for subsequent interaction, compared with passive adsorption.

The coupling was done stepwise to prevent AFP interlinkage and excess DSS was used to prevent coupling of both DSS ends to the surface. Brief activation (one hour) with DSS limited hydrolysis of the active NHS-groups.

A. Activation with DSS

- Prepare three plates, A) CovaLink, B) MaxiSorp and C) PolySorp™.
- Add 100µL DSS solution to half of the wells in plate A. Cover the wells.
- No activation is attempted for MaxiSorp and PolySorp.
- Incubate for one hour at room temperature.

B. Wash

- Empty the wells and wash three times with distilled water.

C. Incubation

- Empty the wells.
- Add 100µL AFP solution to all the PolySorp, MaxiSorp and CovaLink NH wells, cover the wells and incubate overnight at room temperature.

D. Casein blocking

- Empty the wells, add 100µL casein solution and leave for 15 minutes.

E. Wash

- Empty the wells and wash three times with distilled water.

F. Conjugate Incubation

- Empty the wells.
- Add 100µL conjugate solution to all the wells, cover the wells, and incubate for two hours at room temperature.

G. Wash

- Empty the wells and wash three times with distilled water.

H. Substrate Reaction

- Empty the wells.
- Add substrate solution, 100µL well. Wait for color development and stop the reaction by adding 1M H_2SO_4, 100µL/well.
- Read O.D. of wells at 490nm.

I. Results

- The result (Fig. 7) demonstrated that more AFP can be detected on DSS-activated CovaLink NH than on MaxiSorp, PolySorp, or CovaLink NH without DSS, where only passive binding is possible.

J. Conclusion

- DSS activated CovaLink NH is suitable for binding large protein antigens with special significance for molecules which bind weakly by physical adsorption.

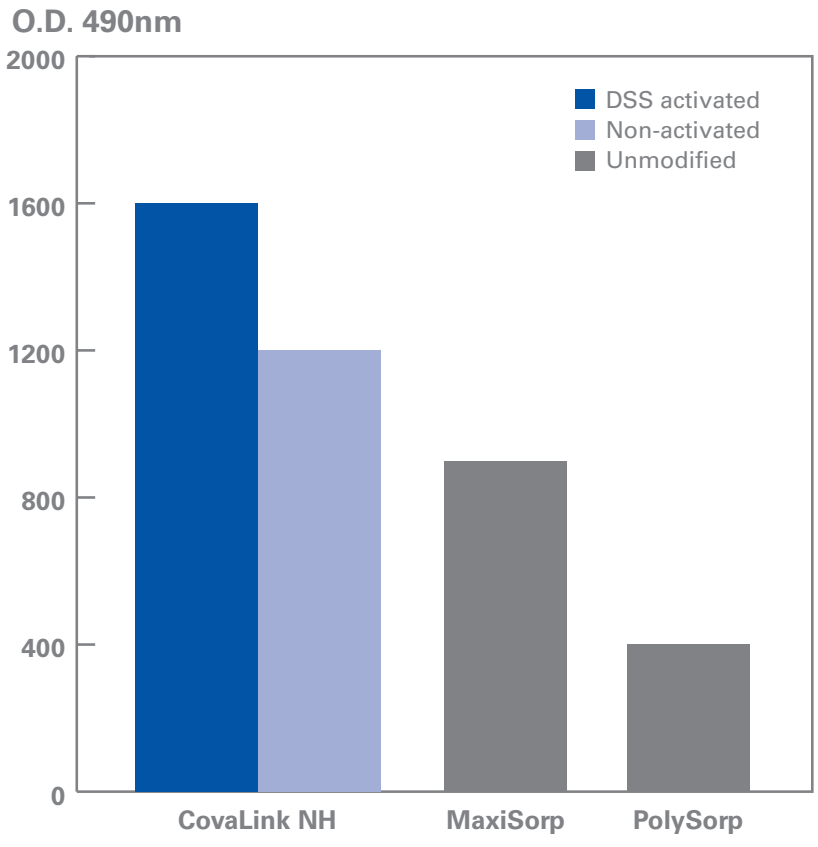


Fig. 7.
Comparison of signals from AFP immobilization on PolySorp, MaxiSorp and unactivated or DSS-activated CovaLink NH, showing that the largest signals was obtained on the latter surface.

Coupling of peptides to CovaLink NH via their carboxylic groups

Formation of amide bonds between carboxylic acids and amines is generated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) which activates the carboxylate by forming an O-acylurea. However, when the reaction is carried out in an aqueous solution the compound is subject to hydrolysis which can significantly limit the yield.

It has been demonstrated that a more hydrolysis resistant active ester can be made by adding sulfo-N-hydroxysuccinimide (sulfo-NHS). The O-acylurea activated ester will react with sulfo-NHS, forming a more stable succinimidyl activated ester (Staros, 1986), e.g. an activated peptide (Fig. 8).

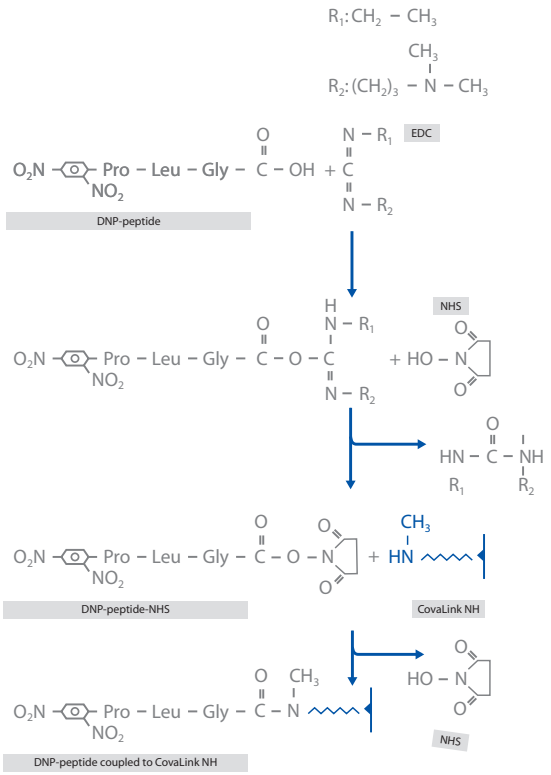


Fig. 8.
Reaction scheme for immobilization of DNP-labeled tri-peptide. See text for further information.

Preparation of reagents and buffers

Material

- Solid Phase: CovaLink NH, MaxiSorp, PolySorp
- Dinitrophenyl-Peptide (DNP-Pro-Leu-Gly)
- Dimethyl sulfoxide (DMSO)
- 1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC)
- Sulfo-N-hydroxysuccinimide (sulfo-NHS)
- Rabbit anti-DNP antibody horseradish peroxidase conjugate (Ra DNP-HRP)
- Ortho-phenylenediamine dihydrochloride (OPD)
- Hydrogen Peroxide (H_2O_2), 30%

DNP peptide stock solution

DNP peptide 14.4mg
Distilled water 0.4mL
DMSO 0.6mL

DNP peptide/NHS solution

DNP peptide stock solution .. 0.5mL
Sulfo-NHS 1.84mg
Distilled water ..... ad 10mL
Note: Use fresh solution

EDC solution

EDC 12.3mg
Distilled water ..... ad 10mL
Note: Use fresh solution

Conjugate Solution

Ra DNP-HRP in CovaBuffer.....
.....2.6µg/mL
Note: Use fresh solution

Substrate solution

OPD 60mg
H_2O_2 (30%) 50µL
Citrate-phosphate buffer.....
..... ad 100mL
Note: Use fresh solution and keep dark

Phosphate Buffered Saline (PBS)

0.15M, pH 7.2
NaCl 8.0g
KCl 0.20g
$Na_2HPO_4 \cdot 2H_20$ 1.15g
KH_2PO_4 ..... 0.20g
Distilled water ..... ad 1000mL
Adjust to pH 7.2 with HCl/NaOH

Citrate-Phosphate buffer

0.1M, pH 5.0
Citric Acid, H_20 ..... 7.3g
$Na_2HPO_4 \cdot 2H_20$ 11.86g
Distilled water ..... ad 1000mL
Adjust to pH 5.0 with HCl/NaOH

CovaBuffer

NaCl 116.9g
$MgSO_4 \cdot 7H_20$ 10.0g
Tween 20 ..... 0.5mL
PBS ..... ad 1000mL

Example 4

The purpose of this experiment was to demonstrate that a tri-peptide, barely detectable on MaxiSorp and PolySorp, can be detected when bound to CovaLink NH. A tri-peptide, Pro-Leu-Gly, which has only terminal amino and carboxylic groups was used. The terminal proline amino group was labeled with dinitrophenol (DNP), partly to avoid peptide interlinkage, and partly to allow peptide detection by anti-DNP antibody (Fig. 9).

A. Incubation

- Prepare three plates, A) CovaLink, B) MaxiSorp and C) PolySorp as follows:
- Add 100µL DNP peptide/NHS solution to each well in column 2.
- Add 50µL distilled water to all other wells.
  Prepare dilution series by transferring 50µL from the wells in column 2 to column 3, mix, transfer 50µL from column 3 to column 4, mix, etc. After mixing discard 50µL from the wells in column 12.
- Start reaction by adding 50µL EDC solution to all wells. Cover the plates.
- Incubate for two hours at room temperature.

B. Wash

- Empty the wells and wash three times with CovaBuffer.
- Keep the buffer in the wells for 15 minutes after the third wash.

C. Conjugate Incubation

- Empty the wells.
- Add 100µL conjugate solution to each well. Incubate for one hour at room temperature.

D. Wash

- As in B above.

E. Substrate Reaction

- Empty wells.
- Add substrate solution, 100µL/well.
- Wait for color development. To stop the reaction add 1M H_2SO_4 100 µL/well.
- Read O.D. of wells at 490nm.

F. Results

- On the CovaLink NH a significant increase in signal was observed by adding carbodiimide, indicating that covalent binding took place. The presence of carbodiimide on MaxiSorp or PolySorp has no effect.
- It is interesting to note the level of binding of the peptide on CovaLink NH in the absence of carbodiimide (Fig. 10).
  This increase can be explained by the passive adsorption of the peptide on the linker arms of the surface.

G. Conclusion

- From this example it can be seen that CovaLink NH can be recommended for the immobilization of small peptides without the use of a carrier in place of MaxiSorp or PolySorp.

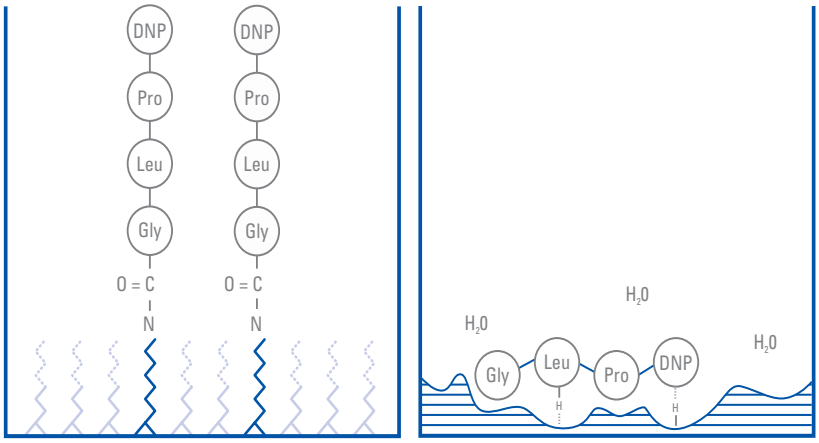


Fig. 9.
Covalent binding of peptide to CovaLink NH (left) and physical adsorption of peptide to MaxiSorp (right).

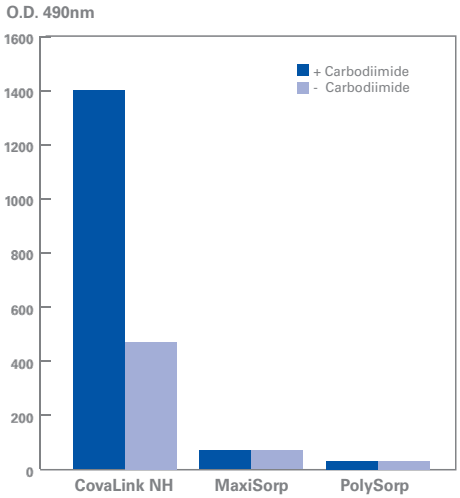


Fig. 10.
Illustrates the difference in binding on the three surfaces tested.
The observed difference can be explained if the size of the peptide is considered. (Fig. 9).
On the CovaLink NH the small peptide is bound via the carboxylic group to the secondary amino group.
On MaxiSorp or PolySorp either the peptide does not adsorb or the molecule is adsorbed, but the antigen determinant is undetectable probably due to its inaccessibility to the antibody.

Coupling a hapten having a Carboxylic group to CovaLink NH

Binding of molecules to CovaLink NH can occur through the interaction between a carboxylic group on the molecule and the grafted NH group by the formation of an amide bond.

The formation of this linkage is promoted by the action of carbodiimide and Sulfo-NHS.

Fig. 11 shows the reaction scheme for the coupling of the hapten biotin through its available carboxylic group.

Preparation of reagents and buffers

Material

- Solid Phase: CovaLink NH, MaxiSorp
- d-Biotin
- Dimethyl sulfoxide (DMSO)
- 1-Ethyl-3 (3-dimethylaminopropyl)-carbodiimide (EDC)
- Sulfo-N-hydroxysuccinimide (sulfo-NHS)
- Avidin
- Avidin horseradish peroxidase conjugate (Avidin-HRP)
- Ortho-phenylenediamine dihydrochloride (OPD)
- Hydrogen Peroxide (H_2O_2), 30%

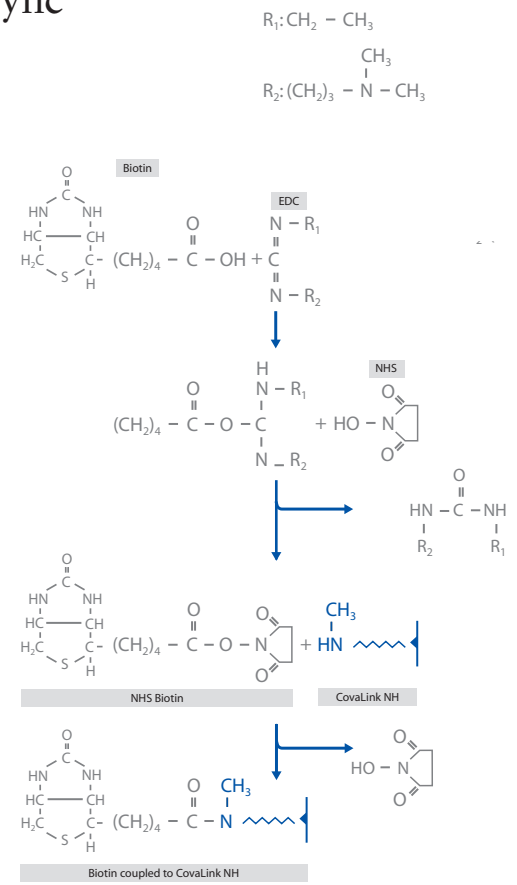


Fig. 11.
Reaction scheme for immobilization of biotin. Carbodiimide generates the formation of amide bonds between carboxylic acids and amines by activating the carboxylate to form an O-acylurea. When the reaction in carried out in an aqueous milieu a more hydrolysis-resistant active ester can be made by adding N-hydroxysuccinimide (NHS).

Biotin stock solution

d-Biotin.....7.8mg
Distilled water 0.4mL
DMSO 0.6mL

Biotin/NHS solution

Biotin stock solution.....0.5mL
Sulfo-NHS.....1.83mg
Distilled water ..... ad 10mL
Note: Use fresh solution 10mL/plate required

EDC solution

EDC.....5.8mg
Distilled water ..... ad 10mL
Note: Use fresh solution

Avidin Mix

Avidin. 400µg
Avidin-HRP..... 13µg
Cova Buffer ..... ad 100mL

Substrate solution

H_2O_2 (30%) 50µL
OPD..... 60mg
Citrate-phosphate buffer.....
..... ad 100mL
Note: Use fresh solution and keep dark

Phosphate Buffered Saline (PBS)

0.15M, pH 7.2
NaCl 8.0g
KCl 0.2g
$Na_2HPO_4 \cdot 2H_2O$ 1.15g
KH_2PO_4 ..... 0.2g
Distilled water .....ad 1000mL
Adjust to pH 7.2 with HCl/NaOH

CovaBuffer

NaCl 116.9g
$MgSO_4 \cdot 7H_2O$ 10.0g
Tween 20 .....0.5mL
PBS .....ad 1000mL

Citrate-Phosphate buffer

0.1M, pH 5.0
Citric Acid, ($C_6H_8O_7 \cdot H_2O$).....7.3g
$Na_2HPO_4 \cdot 2H_2O$ 11.86g
Distilled water .....ad 1000mL
Adjust to pH 5.0 with HCl/NaOH

Example 5
Coupling biotin to CovaLink NH

This experiment was carried out in order to compare the binding of a hapten to MaxiSorp and to CovaLink NH.

A. Incubation

- Set up one CovaLink and one MaxiSorp plate as follows:
- Add 50µL of distilled water to each well, apart from those in column 2.
  Add 100mL Biotin/NHS solution to a wells in column 2.
- Make a solution series by transferring 50µL from the wells in column 2 to column 3, mix, transfer 50µL from column 3 to column 4, mix, etc.
- After mixing discard 50µL from the wells in column 12.
- Start reaction by adding 50µL EDC solution to each well. In control experiment add a 50µL distilled water instead of EDC. Cover the wells.
- Incubate at room temperature for 90 minutes.

B. Wash

- Empty the wells. Wash three times with CovaBuffer. Keep the buffer in the wells for 15 minutes after the last wash.

C. Conjugate Incubation

- Empty the wells.
- Add 100µL Avidin mix to each well.
  Incubate at room temperature for two hours.

D. Wash

- Empty the wells. Wash three times with CovaBuffer. Keep the buffer in the wells for 15 minutes after the last wash.

E. Substrate Reaction

- Empty the wells.
- Add substrate solution, 100µL/well.
- Wait for color development and stop the reaction by adding 1M H_2SO_4, 100µL/well. Read O.D. of wells at 490nm.

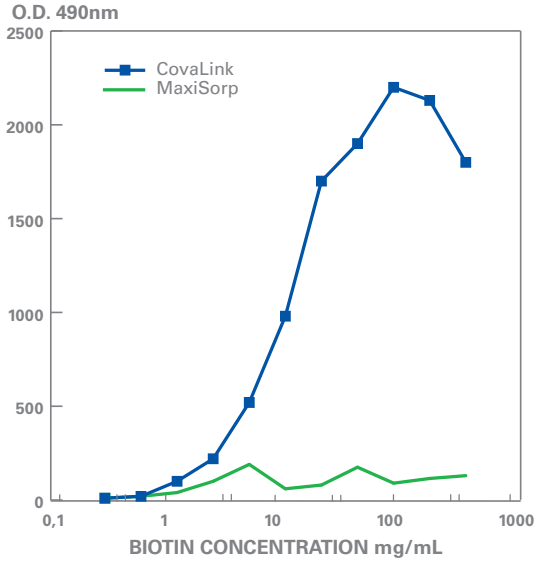


Fig. 12.
This result illustrates the difference in amount of biotin bound to CovaLink NH and to MaxiSorp.

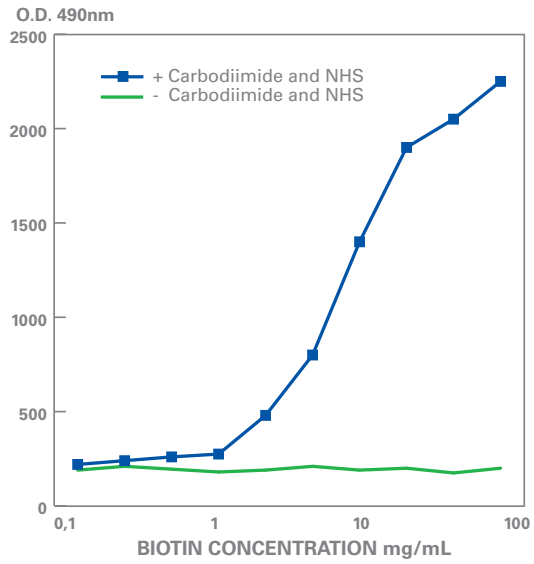


Fig. 13.
This result illustrates that the addition of carbodiimide and NSH is essential for the binding of biotin to the secondary amino groups on the CovaLink NH surface.

F. Result

- The correlation between biotin concentration and the signal measured in the EDC-activated CovaLink plate is shown in Fig. 12.

G. Conclusion

- Covalent coupling has taken place between the carboxylic group on the biotin and the secondary amino group grafted on the CovaLink NH.

Example 6
The effect of carbodiimide activation

The purpose of this example is to illustrate effect of carbodiimide activation on the immobilization of biotin on CovaLink NH.

The procedure was similar to experiment 5 except that two CovaLink plates were used in one of which carbodiimide and sulfo-NHS were omitted in step A, as a control for carbodiimide activation.

The result showed that without added carbodiimide the covalent binding of biotin did not occur (Fig. 13).

Glutaraldehyde activation of the CovaLink Surface

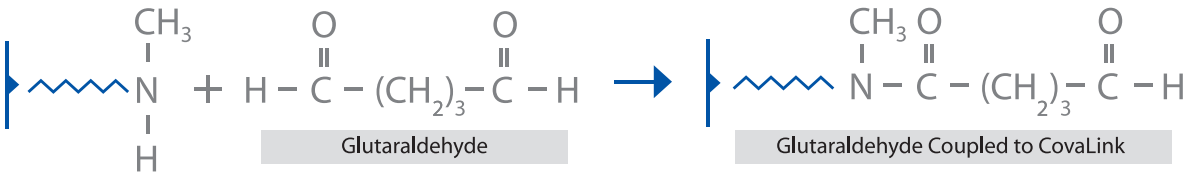


Fig. 14.
Reaction scheme for the activation of the CovaLink NH surface using glutaraldehyde, by replacing the secondary amine with an aldehyde function.

For some applications it may be advantageous to activate the CovaLink surface itself rather than the molecules which are to be bound to the surface (Fig. 14). The CovaLink NH surface can be activated by the action of glutaraldehyde.

This causes the replacement of the secondary amino group with an aldehyde function.

Preparation of reagents and buffers

Materials

- $Na_2HPO_4.2H_2O$
- KH_2PO_4
- Glutaraldehyde
- Thermo Scientific Pierce EZ-Link 5-(biotinamido) pentylamine (5-BP)
- Avidin
- Avidin-Horseradish peroxidase conjugate
- Ortho-phenylenediaminedi-hydrochloride (OPD)
- Hydrogen peroxide (H_2O_2)
- Citric acid $C_6H_8O_7.H_2O$

Solutions

0.05M Na_2HPO_4
$Na_2HPO_4 \cdot 2H_2O$..... 8.89g
Milli-Q water .....ad 1000mL
0.5M KH_2PO_4
KH_2PO_4..... 6.8g
Milli-Q water .....ad 1000mL

Phosphate buffer 0.05M pH 8.2

Remove 3mL of Na_2HPO_4 stock solution and add 3mL of the KH_2PO_4 stock solution. Mix and adjust to pH 8.2 using HCl or NaOH.

10x Citrate-Phosphate Buffer

$C_6H_8O_7 \cdot H_2O$ 73g
$Na_2HPO_4 \cdot 2H_2O$ 118.6g
Milli-Q water .....ad 1000mL
Adjust to pH 5.0 using concentrated NaOH solution

HRP-Avidin conjugate/Avidin mix

(0.065µg/mL / 4µg/mL)

Glutaraldehyde

Solutions were prepared at 5%, 2.5% 1.25% and 0% using phosphate buffer.

5-(BP)

Was prepared at a concentration of 20 µg/mL and serially diluted using phosphate buffer.

CovaBuffer

NaCl 116.9g
$MgSO_4 \cdot 7\ H_20$ 10.0g
Tween 20 .....0.5mL
PBS .....ad 1000mL

OPD Mix

10x Citrate-Phosphate buffer .....
.....ad 1000mL
OPD Tablets 2x30mg
30% H_20_2 .....50uL
Milli-Q-Water..... ad 100mL
Must be made fresh daily

Stop Solution

1M H_2SO_4

Example 7

The purpose of this example is to find the concentration of glutaraldehyde required to generate a surface having an optimal number of aldehyde functional groups for subsequent reaction.

A. Activation of CovaLink surface

- Four plates were used, one for each concentration of glutaraldehyde. Add 100µL of the same concentration of glutaraldehyde to each well.
- Incubate overnight at 37°C.

B. Wash

- Empty the wells and wash three times with phosphate buffer.

C. Coupling to activated CovaLink surface

- Empty the wells.
- Add 100µL of the phosphate buffer to all wells except those in column 2.
- Add 200µL of 5-BP solution to each well in column 2.
- Make a 1:2 serial dilution by taking 100µL from each well in column 2 and transferring it to column 3. Mix and repeat the process in each subsequent column. Finally, after mixing discard 100µL from each well in column 12.
- Cover the plates with sealing tape.

D. Incubation

- Incubate three hours at 37°C.

E. Wash

- Empty the wells and wash three times with CovaBuffer.

F. Conjugate incubation

- Empty the wells.
- Add 100µL of HRP conjugate Avidin mix to each well.
- Incubate two hours at room temperature.

G. Wash as in E

H. Substrate reaction

- Empty the wells.
- Add 100µL of OPD mix to each well. Wait for color development
- Add 100µL of 1M H_2SO_4 per well to stop the reaction.
- Read OD of wells at 490nm.

I. Result

- A concentration of 1.25% glutaraldehyde is sufficient to give the maximum binding of 5-BP (Fig. 15).

J. Conclusion

- The activation of the CovaLink surface using glutaraldehyde, allows the subsequent binding of molecules with a reactive NH group.

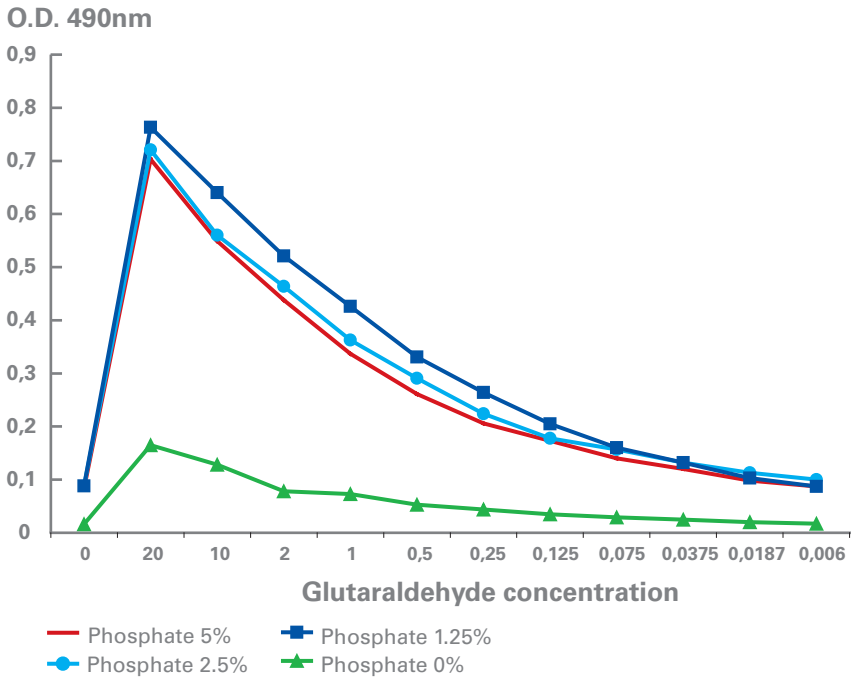


Fig. 15.
Shows OD signal as a function of the concentration of 5-BP on a glutaraldehyde activated CovaLink NH surface.

Trouble shooting guide

Problem	Direct Coupling of NHS-activated molecules	Indirect Coupling using Bifunctional Linker	Direct Coupling using Carbodiimide	Coupling using Glutaraldehyde
No signal	• Make stock solution in DMSO, and dilute immediately before use. • Check buffer pH, should be between 8 or 10. • Avoid using detergent in coupling buffer. • Check that the detection system is working, e.g. that the substrate is properly made.	• Solution of Bifunctional linker should be freshly made. Use 50% DMSO in the working solution. • Check activation time, should be between 0.5 and 2 hours. • Check linker concentration, should be approximately 10µg/well. • Check that no detergent is used in the coupling step. • Check concentration of molecule to be immobilized, should be approximately 10µg/well.	• Check that the molecule to be immobilized contains a group that can be activated, e.g. a carboxylic group. • Solution of carbodiimide should be freshly made. • Check concentration of carbodiimide, should be approximately 200mMol/well. • Check concentration of molecule to be immobilized, should be approximately 10µg/well.	• The amino containing molecule to be bound must be stored dry, e.g. using silica gel. • Avoid buffers containing glycine and Tris since they will react with glutaraldehyde. Phosphate, carbonate and borate buffers are acceptable at alkaline pH and a molarity ranging from 0.01-0.1M. • Check that the detection system is working correctly.
No improvement in signal compared to other plastic supports	• Check for unspecific coupling of conjugate. • Optimise concentration of molecules being immobilized.	• Large molecules that easily adsorb to conventional plastic supports might not show increased signal on CovaLink NH.	• Optimize concentration of molecules to be immobilized. • Optimize temperature and coupling time.	• Optimize the concentration of glutaraldehyde and the amino containing molecule. • Optimize incubation times and temperatures.
Signal ok, but high background is observed	• Check that suspension of activated molecules is fresh. Increase ionic strength of washing buffer, e.g. use 2M NaCl. • Use CovaBuffer for washing, allow microwells to stand with buffer between aspirations. • Block free binding sites, e.g. using 1% dry milk powder in PBS or 0.5% BSA in PBS, not do use detergent before blocking.	• Use CovaBuffer in washing steps. Increase ionic strength of washing buffer and allow the microwells to stand with washing-buffer between aspirations. • Block free binding site, using 1% dry skimmed milk powder or 0.5% BSA in PBS. Avoid using detergent before blocking.	• Use CovaBuffer in washing steps. Increase ionic strength of washing buffer and let microwells stand with washing solution between aspirations. • Block free binding sites, using 5% skimed milk powder or 0.1% BSA in PBS. • Do not use detergent before blocking	• Optimize the buffer pH. • Change buffer to one of the three alternatives given above. • Increase the ionic strength in the buffer used in the incubation step and/or in the washing step. • Include a blocking step, e.g. using 0.5% BSA, 1% dextrose or equivalent depending on system.

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