NL.050

NaveniLink™

1. Overview

NaveniLink enables simple and rapid conjugation of oligonucleotide arms to antibodies. The kit is compatible with NaveniFlex Cell, NaveniFlex Tissue and NaveniBright.

The kit includes two distinct oligonucleotide arms ready to conjugate 50 µg of antibody each. The protocol involves only a few steps, requires minimal hands-on time and doesn't require any expertise in antibody conjugation or oligonucleotide sourcing.

Please find antibody buffer considerations and compatibility below.

2. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental

protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.

- To ensure success with your Proximity Ligation Assay, antibodies must be carefully selected, please see FAQs for further guidance.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

3. Storage and Stability

- Store the kit at -20°C upon receipt.

Lyophilized components are hygroscopic.

Kits are intentionally shipped at ambient temperature with silica gel to avoid exposure to moisture. Upon receipt, store the kit frozen and protect from moisture. Before opening the outer

container, allow the lyophilized components to reach room temperature to minimize condensation.

4. Limitations

Assay kit intended for research use only. Not for use in diagnostic procedures.

Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

5. Materials Supplied

Storage: -20°C.

FREEZE 

Item	Quantity
Modifier	1 vial
Oligo arm 1 and Oligo arm 2	2 vials
Quencher	1 vial

6. Buffer Considerations

Recommended antibody buffer conditions and components.

Buffer Components	Compability
pH	**6.5-8.5**
Amine free buffers (e.g. MOPS, MES, HEPES, PBS)	Ok
Non-buffering salts (e.g. sodium chloride)	Ok

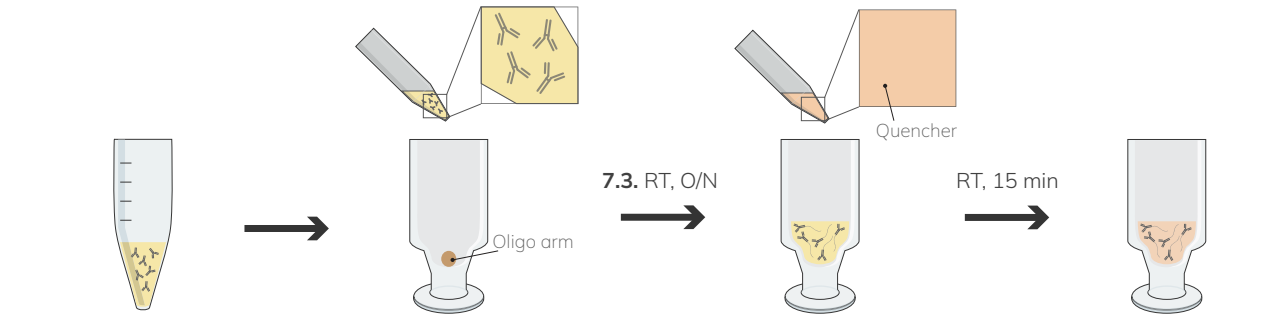
Buffer Components	Compability
Chelating agents (e.g. EDTA)	Ok
Sugars	Ok
Glycerol	Not tested
Thiomersal/ Thimerosal	Not recommended
Merthiolate	Not recommended
Sodium azide	≤ 0.25%
BSA	Not recommended
Gelatin	Not recommended
Tris	Not recommended
Glycine	Not recommended
ProClin 300	Not recommended
Borate buffer	Not tested
Nucleophilic components (e.g. amino acids, ethanolamine, mercaptoethanol or DTT)	Not recommended

Note: Compounds above marked ‘not tested’ yet are expected to be tolerated but no confirmatory data is available at this time. Please note that this table refers to expected impact in conjugation.

You should also give due consideration to effects of these substances in your application, especially if the optional clean-up step is not required.

7. Assay Procedure

Equilibrate all materials and prepared reagents to room temperature prior to use. Antibodies should be 1 mg/mL in a suitable buffer (See Section 6 for compatible substances). Antibodies that are <1 mg/mL should ideally be concentrated.



7.1. Prepare Antibody/ Modifier mix

7.2. Add Antibody/Modifier mix to Oligo arm

7.4. Add Quencher to Conjugate

7.5. Ready-to-use Conjugate

7.1. Dilute your antibody to 1 mg/mL (See Section 6 for buffer compatibility) and add 5 µL of Modifier to 50 µL of antibody.

7.2. Remove the cap from a vial of Oligo arm and pipette 55 µL of antibody/Modifier mix directly onto the lyophilized material. Resuspend gently by withdrawing and re-dispensing the liquid until the powder dissolves.

7.3. Place the cap back on the vial and leave the vial standing at room temperature (20-25°C) overnight. It is convenient to set up reactions late in the day though the exact set-up time is not critical.

7.4. At the end of the overnight incubation, add 5 µL of Quencher and leave at room temperature for 15 minutes.

7.5. The conjugate is now ready.

Note: There are three optional steps – (i) Clean-up of the conjugate to remove free oligo, (ii) Conjugate concentration measurement and (iii) SDS-PAGE gel analysis of a small aliquot of the conjugate. Either or all steps may be appropriate, and these are explained in Sections 10, 11 and 12, respectively.

8. Storage of Conjugates

For any new conjugate, initial storage at 4°C is recommended. A preservative may be desirable for long-term storage. Other storage conditions (e.g. frozen at -70°C or at -20°C with 50% glycerol) may also be satisfactory. We recommend to store the conjugates in PBS with 4mM EDTA and 0,03% proclin and store at +4°C. However, the best conditions for any new conjugates must be determined experimentally.

You should also take into account of how the conjugate will be used and avoid adding substances that will need to be removed later, resulting in inevitable loss of conjugate.

9. Varying the degree of labeling

Antibodies that are too concentrated can be diluted in an amine-free buffer (see Section 6) to 1 mg/mL. In most cases, you will therefore be adding 55 µL (50 µL + 5 µL, from step 7.2) to the oligo reaction vial.

As the required degree of labeling may be application dependent, the quantity of the antibody may be varied to fine tune the conjugate performance. The recommended range for exploration is 25 – 100 µg of antibody in 25 – 100 µL volume (or 27.5 – 110 µL volume with Modifier). Some examples showing the effect of changing reaction conditions are given in Section 12.

10. Conjugate clean-up procedure (Optional)

Note: If you intend to do SDS-PAGE before carrying out clean-up, remove 1 µL of conjugate prior to the optional clean-up.

10.1. Equilibrate the filter with 500 µL 1x PBS.

10.2. Centrifuge for 5 mins at 14,000 xg.

10.3. Discard the flow through.

10.4. Add the conjugate sample to the filter and fill up to a total volume of 500 µL with 1xPBS.

10.5. Centrifuge for 5 mins at 14,000 xg.

10.6. Discard the flow through.

10.7. Add 450 µL 1x PBS to the filter.

10.8. Centrifuge for 5 mins at 14,000 xg.

10.9. Repeat steps 10.7 and 10.8. for additional 7 times (8 washes in total).

10.10. After final wash, remove flow through and invert the filter into a clean tube.

10.11. Centrifuge for 2 mins at 1,000 xg to recover the sample.

10.12. Dilute the sample (if necessary) to 50 µL total.

10.13. Store the cleaned up conjugate at 4°C.

11. Conjugate concentration and dilution (Optional)

Concentration of resuspended conjugate after clean-up can be determined using a BCA

assay with a relevant IgG standard curve for quantification.

11.1. Use the determined concentration to dilute conjugate to desired concentration using a buffer appropriate for your application.

11.2. Alternatively, if you do not wish to determine an exact concentration, an assumption of 50-60% recovery after clean-up can be used, however the exact recovery will vary between conjugates.

12. SDS-PAGE (Optional)

SDS-PAGE may be used to confirm successful conjugation. To carry out SDS gel analysis, remove 1 µL of conjugate prior to the optional clean-up and add to 10 µL of either reducing or non-reducing sample buffer. Repeat with the cleaned up conjugate. Run the samples on a gradient gel (a 4-12% gel is ideal).

Optional: Stain the gel for DNA to estimate clean-up efficiency.

12.1. Reducing gels have the advantage that antibody heavy (H) and light (L) chains migrate some distance in the gel and the band shifts caused by the attachment of oligos are quite

pronounced. Reducing gels also provide an indication of how many oligos have been attached to the H and L chains. Please note that this number will be in a range, not a single number. Some heavy and light chains will also remain unlabeled, even if an IgG has reacted with several oligos.

12.2. On non-reducing gels, the four antibody chains migrate as a single unit and an IgG molecule with just one oligo attached will always exhibit a band shift. (On a reducing gel one chain would exhibit a band shift and three would not). A non-reducing gel is therefore very useful for demonstrating the absence of unlabeled antibodies in conjugates, however, the resolution of the various bands is less than on a reducing gel and it is particularly important not to overload non-reducing gels.

12.3. An example is shown in Fig. 1 with a polyclonal antibody. The gel has been deliberately overloaded (~4 µg of antibody conjugate and IgG) to illustrate the band patterns more clearly, especially the quantitatively minor bands in the reducing half of the gel (Fig. 1, Tracks 3 and 4) i.e. those with two or more oligos attached. On the non-reducing half of the gel (Tracks 1 and 2), the block of overlapping conjugate bands (Track

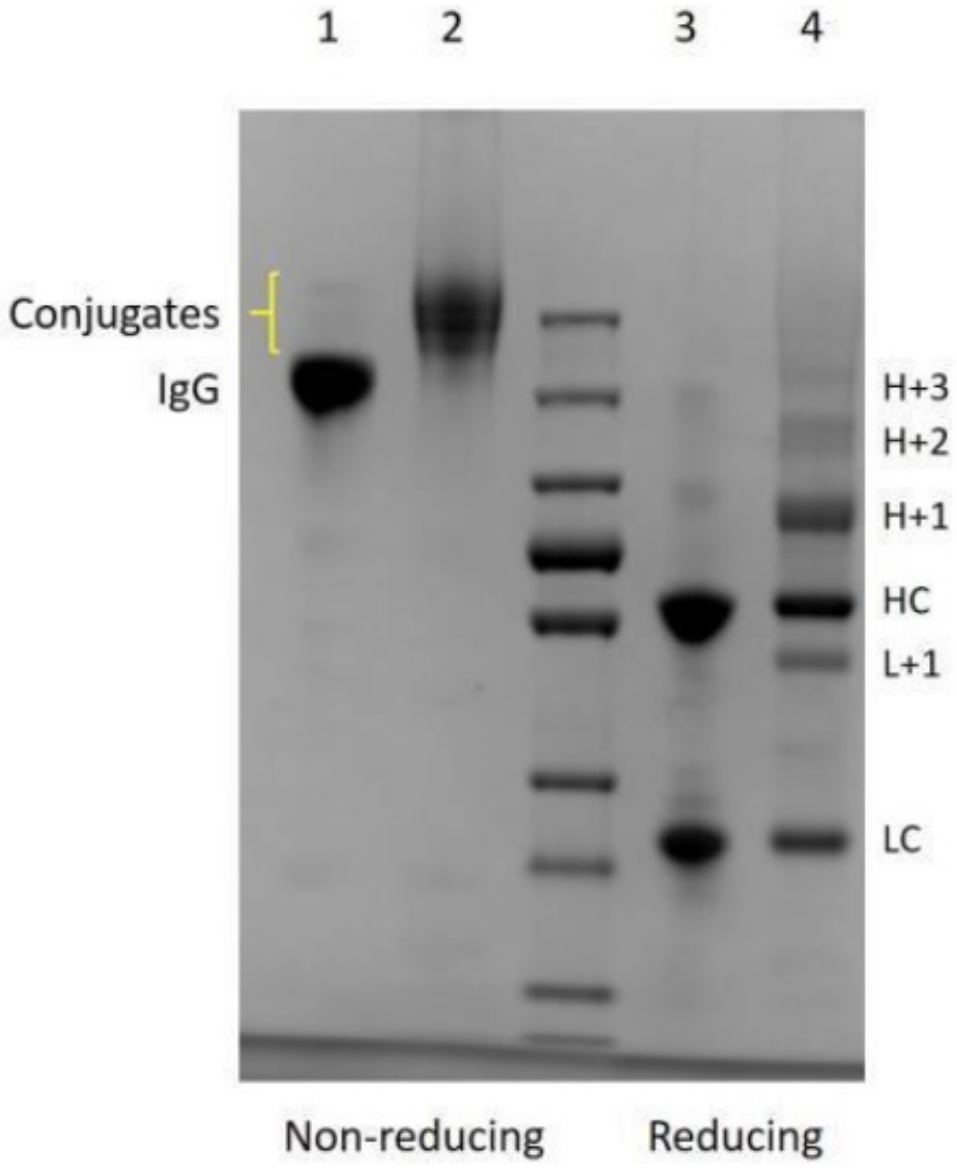


Fig 1.

2) clearly has lower mobility than the reference IgG, indicating that no unlabeled antibodies are present in the conjugate.

Sometimes discrete conjugate bands will be seen on non-reducing gels and this is dependent on the type of antibody, quality of the gradient gel and the amount of sample loaded. Irrespective of the resolution within the conjugate bands, one may deduce from the extent of the band shift

compared with an IgG reference band whether any unlabeled antibodies are present in the conjugate.

12.4. Discrete conjugate bands are more commonly seen with monoclonal antibodies and with lower loadings than those used above. In Fig. 2, Tracks 2 and 6 were loaded with 2 µg of conjugate produced under non-reducing or reducing conditions. At this lower loading, the H+2 band is still visible but H+3 (not labeled

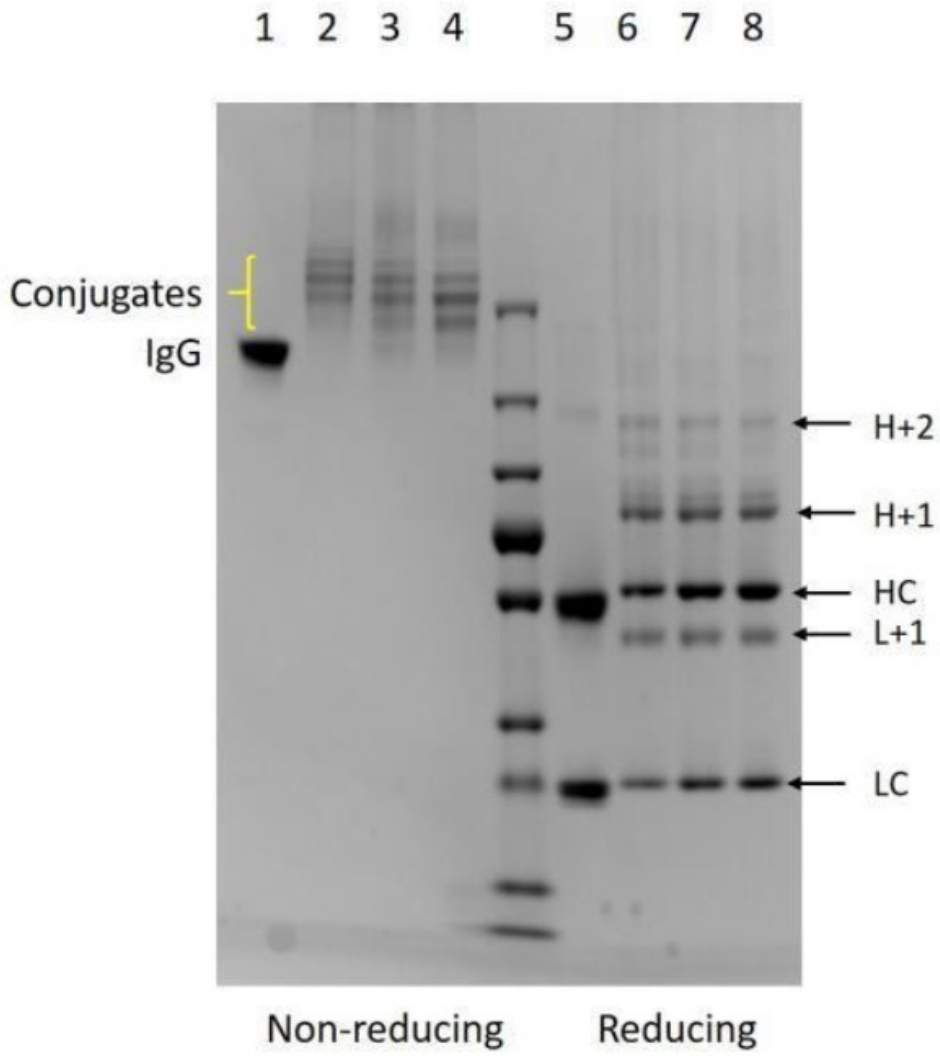


Fig 2.

on Fig. 2) is faint. On the non-reducing gel, the number of oligos attached to IgGs is readily determined. Further commentary and details of the other samples (Tracks 3, 4, 7 and 8) are given below. A magnified view of the IgG conjugates in Fig. 2 is shown in Fig. 3.

12.5. In Fig. 3, track 2 shows no free IgG and very little IgG with 1 oligo attached. Mostly there are 2-5 oligos per IgG (IgG+5 is not marked by an arrow above). In tracks 3 and 4, the conjugation reactions comprised, respectively, 20 µg in 10 µL and 20 µg in 20 µL. Not surprisingly, the labeling

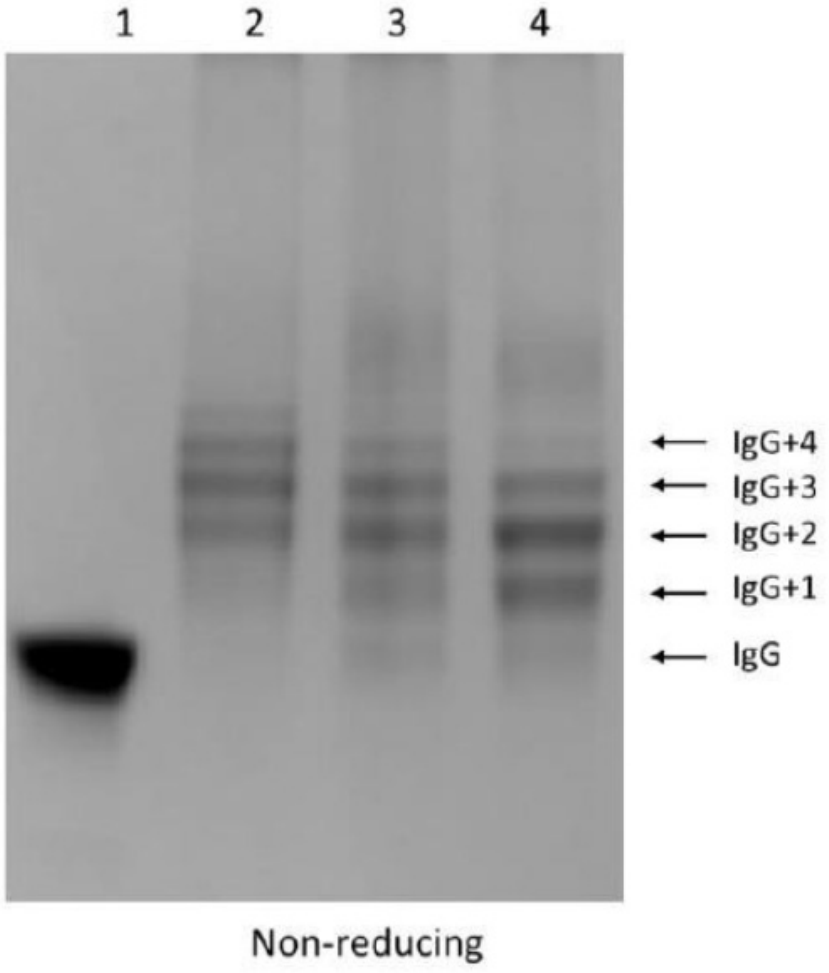


Fig 3.

ensity changes and there are 1- 4 oligos per IgG (but mainly 2-3 oligos) in track 3. In track 4, most conjugates have 1 or 2 oligos. As shown, there are smaller quantities of unlabeled IgG as the average oligo density is reduced.

12.6. The differences among the samples under reducing conditions (Tracks 6, 7 and 8, Fig. 2) are less pronounced and the main observation is increasing H or L chain intensity as conjugation efficiency is reduced. For each application, it may be necessary to determine the gel profile that is associated with best assay performance, and non-reducing gels are generally more useful.

12.7. If only reducing gel data are available, there are still pointers that help with the assessment of conjugation efficiency. One would expect to see bands corresponding to H, H+1, H+2 and H+3. L+1 will be clearly visible and L+2 will be present, although partially obscured by H+1. The H+3 bands are easier to see if a large amount of sample is applied, but this is not always desirable (see below).

12.8. Another useful approach is to run a matched amount of unlabeled antibody. A reduction in the intensity of the H and L chains

in the conjugate sample of around 50% or more (because of the shifting of some of the H and L chains), provides a strong indication, on probability grounds, that most IgG molecules will have at least one oligo attached.

12.9. Finally, with 10 µg scale conjugation, one would probably be reluctant to run more than 1 µL of conjugate routinely on gels. A typical profile for 1 µL (from an 11 µL scale reaction) under both non-reducing and reducing conditions is shown in Fig 4. You may prefer to run 1 µL of sample in just one of these conditions. The non-reduced sample (Track 1) shows clearly that all antibodies are conjugated (in this case mostly with 1-3 oligos). Following reduction, L+1, H+1 and H+2 are clearly visible. H+3 may be absent when only 1 µL of conjugate is analyzed (~0.9 µg) in reducing conditions.

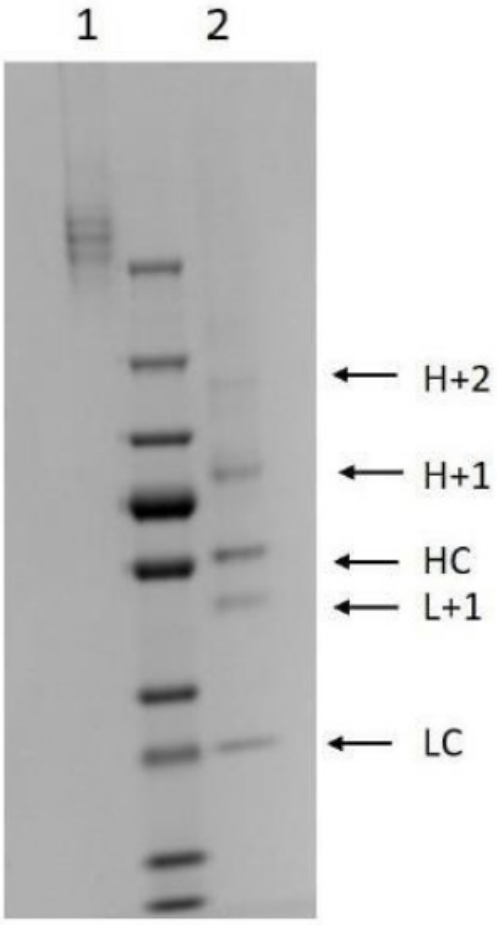


Fig 4.

13. Troubleshooting

My gel has no conjugate bands. What happened?
An absence of conjugate bands almost without exception indicates that the antibody contains interfering substances. Things to watch out for (in addition to substances discussed in Section 6) are whether the antibody is provided as tissue culture supernatant or ascites fluid, each of which must be cleaned up before conjugation.

It is also important to understand that if the mass of protein stated on the vial relates to antibody or to antibody plus other substances e.g. BSA. Finally, you should be wary of antibodies that are sold as '100 tests' or '100 µL', especially if other details are lacking. In these formats, the antibody has usually been heavily diluted.

My antibody is too diluted - what should I do?
Ideally, you should concentrate the sample using spin filters. As there will be some loss of antibody, you will need more than 500 µg to carry out this procedure. Thus, if you only have 500 µg and the concentration is low (e.g. >0.5 mg/mL and <1 mg/mL), it may be better (though not ideal) to perform conjugation under suboptimal conditions. Of course, this could negatively impact assay performance.

Do I need to clean-up my conjugate?

As there are many different applications, only some general guidelines can be given. If free oligo is likely to be problematic, you will need to clean-up the conjugate. However, if the antibody binds to a surface in the application of interest, it may be possible to wash away any free oligo prior to hybridization/amplification and avoid the conjugate clean-up steps in Section 10. Ultimately, you may need to test the conjugate with and without the clean-up steps to determine if some of the processing steps can be avoided.

For more information, or to place an order, visit www.navinci.se/products

Email: contact@navinci.se

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