APPLICATION NOTE

A straightforward method to conjugate antibodies to oligonucleotides – preparation, purification and their applications

Keywords: Antibody Oligonucleotide conjugation | Labelling techniques | proFIRE®

Antibody oligonucleotide conjugates are playing a significant role for a wide range of diagnostic and therapeutic applications. Therefore, there is great interest in using pure antibody oligonucleotide conjugates for protein detection by binding to specific protein targets. To sustain antibody functionality, as well as an efficient conjugate yield and a pure conjugate, it is important to conjugate under optimized physiological reaction conditions with no adverse effects on the antibodies. Using copper-free click chemistry and the unique feature of oligonucleotides, which allows easy functionalization, modifying antibodies with oligonucleotides is a straightforward technique.

In this application note, we introduce a conjugation kit for an easy-to-use and covalent conjugation of antibodies to oligonucleotides with high efficiency and best performance in functional tests.

Background

In recent years, antibody-oligonucleotide conjugates have been used in numerous applications, such as proximity ligation assays, immuno-PCR, DNA-PAINT, CITE-Seq and biosensors (e.g. **switch**SENSE®).[1-7] All these methods have in common their unmet need for precise and pure conjugates. Various methods have been developed to conjugate oligonucleotides to antibodies. Commercial kits are available to produce oligonucleotide-conjugated antibodies, but mostly they involve high costs or simply do not yield a pure product. Here, we present an easy method which can be used to produce antibody-oligonucleotide conjugates for different kinds of applications, including mono-, double-, or triple-conjugates.

The synthesis of oligonucleotide-conjugated antibodies is based on the copper-free click reaction using a strain-promoted alkyne–azide cycloaddition (SPAAC) between dibenzocyclooctyne (DBCO) and an azide, combined with an ion-exchange chromatography purification using the **pro**FIRE® system. The oligonucleotide was attached to the amine groups of an antibody to achieve maximum yield under physiological conditions and maintain binding activity. The SPAAC-based conjugation method is simple, cost-effective, and well suited for the preparation of oligonucleotide-conjugated antibodies for various protein assays.

Here, we present a new conjugation kit tailor-made to perform conjugations of antibodies to oligonucleotides.

Methods and Results

In this Application Note, we describe a straightforward method to conjugate antibodies to oligonucleotides using copper-free click reaction based on strain-promoted alkyne–azide cycloaddition (SPAAC). In SPAAC, cyclooctynes such dibenzocyclooctyne (DBCO) are used to react with azide-functionalized molecules. We have performed two different approaches (Table 1). One way was to attach a DBCO-modified oligonucleotide through a sequential addition of an azide-NHS crosslinker to the antibody. The other way was to attach an azide-modified oligonucleotide through the sequential addition of a DBCO-NHS crosslinker to the antibody. By adding the reactive linker to the oligonucleotide as a first step, we ensured a one-to-one ratio for this reaction, despite an excess of linker. As antibodies contain several amine groups (NH_2), which are distributed throughout the antibody as lysines (Lys) and N-terminal amine groups, it would be much more difficult to ensure a one-to-one ratio between the antibody and the linker when using an excess of the linker. The reactions were always conducted in physiological conditions to prevent adverse effect on the antibody. In both approaches, the oligonucleotide was modified at the 5'-end. In this new kit tailored for the conjugation of antibodies, it is possible to gain different ratios between mono-, double-, and triple-oligonucleotide antibody conjugates, depending on the used molar amounts of reactive linkers vs. oligonucleotides and modified oligonucleotides vs. antibodies, respectively.

	Oligonucleotide	Crosslinker	Antibody
①	DBCO	N_3-PEG_4-NHS	
②	N_3	DBCO-PEG_4-NHS	

Table1 | Used orientations for the conjugation.

The conjugation protocol is very simple, and we started with approach number one. In a first step, the 5'-DBCO modified DNA was incubated with a molar excess of the N_3-PEG_4-NHS crosslinker in a PBS buffer for 20 mins at room temperature. After removing the excess of the crosslinker by using two Zeba™ Desalting Spin columns (ThermoFisher), the modified oligonucleotide was incubated with the therapeutic antibody Remicade® overnight at 4°C. After incubation, the antibody-oligonucleotide conjugate was purified using a **pro**FIRE® instrument. Depending on the length of the oligonucleotide, the correct program was chosen automatically. We used different ratios and found that conjugate peak 1 is significantly higher if the molar amount of reactive linker is tenfold higher than the molar amount of oligonucleotide, and the amount of antibody is equivalent to the molar amount of oligonucleotide (Figure 2).

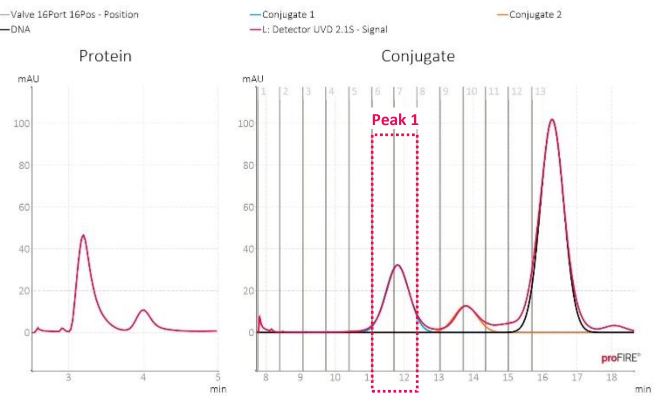

Figure 2 | **pro**FIRE® purification chromatogram of Remicade® conjugated to a 48mer oligonucleotide. Conjugate peak 1 is significantly higher, assuming a 1:1 ratio.

Now we wanted to test if we could produce higher-labeled antibodies as well as ways to control the labeling. If the molar amount of the reactive linker is 25-fold higher compared to the oligonucleotide, and the oligonucleotide is tenfold higher compared to the antibody, the conjugate peak 2 is higher than conjugate peak 1; furthermore, a third peak can be seen (Figure 3), assuming a triple-labeled antibody. By using the **pro**FIRE® instrument, it is easy to separate between the different antibody populations.

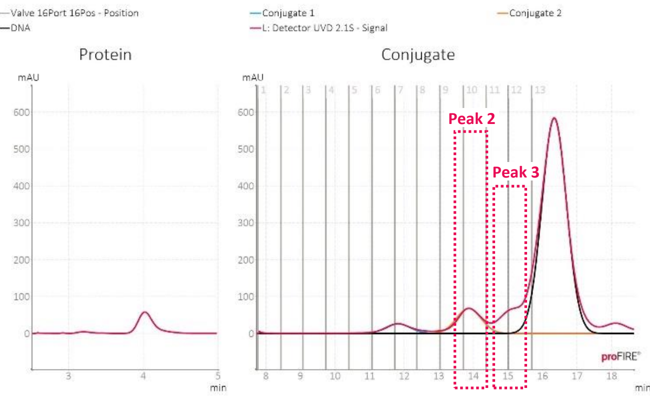

Figure 3 | **pro**FIRE® purification chromatogram of Remicade® conjugated to a higher molar amount of a 48mer oligonucleotide. Conjugate peak 2 is significantly higher, and a new conjugate peak 3 arises.

Depending on the amount of the used oligonucleotide and reactive linker, the ratios of mono- double and triple-oligonucleotide antibody conjugate peaks vary and can be adjusted accordingly. Another finding was that the yield of the antibody oligonucleotide conjugate can be improved when using an excess of the oligonucleotide, but the population of higher-labeled antibodies might increase as well.

In order to prove the mono-, double-, and triple-oligonucleotide conjugated antibodies, we performed a SDS-PAGE with fluorescence detection. By adding a complementary oligonucleotide carrying a fluorescent dye to the purified antibody-oligonucleotide conjugates, the conjugates can be detected easily (Figure 4). Peak 1 clearly shows a single population of a 48mer labelled antibody with a 1:1 ratio. Peak 2 shows a higher molecular weight, assuming a 2:1 ratio. And peak 3 shows an even higher molecular weight, assuming a 3:1 ratio. To show the functionality of the labeled antibodies, Remicade® was immobilized on a **switch**SENSE® biosensor chip and checked for the binding of TNFα (data not shown).

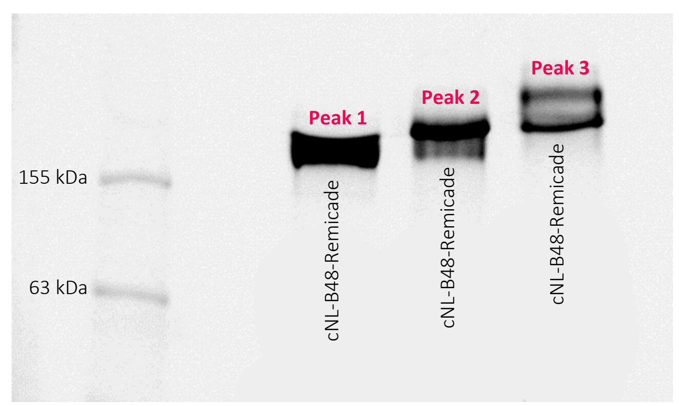

Figure 4 | 7.5% SDS-PAGE gel with fluorescent detection. A fluorescent marker was used in the first lane. Lane 2 corresponds to the peak from the first reaction (tenfold excess of the linker) and lane 3 and 4 corresponds to thepeaks from the second reaction (25-fold excess of the linker).

We repeated all the experiments with the second approach (using an azide-modified oligonucleotide) and found out that the yields are all comparable; therefore, we just went on with the first approach due to practicability.

In order to compare our method with commercially available kits for the prepration of antibody-oligonucleotide conjugates, we used a 30mer control DNA and Remicade® and followed the manufacturer's

protocol for the preparation and purification of the conjugate. After careful preparation, we injected the conjugate product in the **pro**FIRE® instrument to check the purity of the conjugate. The synthesis worked, but the free DNA could not be removed from the conjugate, which is crucial for subsequent experiments (Figure 5**A**). To prove that the purification worked for our method, we injected the conjugate peak 1 again in the **pro**FIRE® instrument, and no impurities of remaining DNA can be detected which shows the purity of the conjugate (Figure 5**B**).

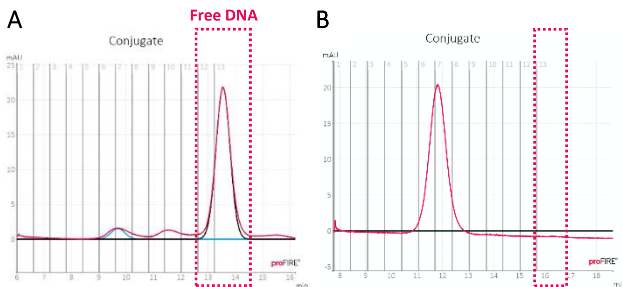

Figure 5 | Comparison chromatograms of two different methods of preparing and purifying conjugates. **A** Used protocol from a competitor. **B** Protocol described here with subsequent **pro**FIRE® purification.

Conclusion

We have presented a new, straightforward way to synthesize antibody-oligonucleotide conjugates. Using different molar ratios of oligonucleotides, reactive linkers, and antibodies, it is now possible to conjugate antibodies covalently to oligonucleotides with high efficiency to get preferential ratios of mono-, double-, and triple-labeled conjugates. The separation of these conjugate peaks was obtained with the **pro**FIRE®. Furthermore, this method can be used to synthesize antibody-oligonucleotide conjugates for a wide range of different applications, including bioanalytical chemistry, molecular diagnostics, or bionanotechnology. The kit can be purchased from Dynamic Biosensors (order no. PF-NH2-3) and can be used for the preparation of mono-labeled antibodies with oligonucleotides with a length from 20-100 bases.

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