PROTOCOL

Thunder-Link® PLUS Oligo Conjugation System

Applicable to: 425-0000 1 reaction plus control (a single reaction to conjugate 100µg antibody)
425-0300 3 reaction plus control (each reaction to conjugate 100µg antibody)

Release 1. © EXPEDEON. 13/03/2018

INTRODUCTION

Antibody-oligonucleotide conjugates are the next generation of tools in biomarker detection, overcoming sensitivity and linear range issues often encountered with standard antibody labels. Antibody-oligonucleotide conjugates also have the potential to be the platform tool in multiplexed protein diagnostic assays. The Thunder-Link® oligo conjugation system allows antibody oligo conjugates to be generated very easily and efficiently, while the inclusion of positive controls enables the end user to confirm that the conjugation chemistry is working correctly.

SHIPPING AND STORAGE

This kit is shipped at ambient temperature. Upon receipt, store the Activation Reagents and buffers pack at 2-8°C and the Clean Up Reagent and columns at room temperature.

KIT CONTENTS

- 2 or 4 glass vials of Thunder-Link® PLUS Oligo Activation Reagent (depending on pack size)
- 2 or 4 glass vials of Thunder-Link® PLUS Antibody Activation Reagent (depending on pack size)
- 1 vial of freeze dried control oligo
- 1 vial of freeze dried control antibody
- 4 or 8 separating columns (depending on pack size)
- 1 x Thunder-Link® PLUS Wash Buffer (80 ml or 160 ml, depending on pack size)
- 1 bottle of Thunder-Link® PLUS Conjugate Clean Up Reagent
- 1 or 2 vials of Thunder-Link® PLUS Antibody Suspension Buffer (depending on pack size)

** Warm the Thunder-Link® PLUS Conjugate Clean Up Reagent by placing the tube in warm water (not warmer than 40°C) for 10 minutes, then shaking to re-dissolve the contents. Once dissolved, maintain the tube at ~22°C to prevent any crystal formation before use.*

If the sample does not dissolve completely, spin the sample in a bench top micro-centrifuge, at a recommended maximum speed of 13,000g for 1 minute. Use the supernatant as described in the protocol.

BUFFER CONSIDERATIONS

Buffer Components	Oligonucleotide buffer	Antibody buffer
pH	6-8	7-9
Amine free buffer *(ideally phosphate buffer)*	✓	✓
Non-buffering salts *(e.g. sodium chloride)*	✓	✓
Chelating agents *(e.g.EDTA)*	✓	✓
Sugars	✓	✓
Glycerol	<50%	<50%
Thiomersal	✗	✗
Thimerosal	✗	✗
Merthiolate	✗	✗
Sodium Azide*	<0.1%	<0.1%
BSA*	<0.1%	<0.1%
Gelatin*	<0.1%	<0.1%
Tris	✗	<20mM
Glycine	✗	✗
Primary amines *(e.g. amino acids)*	✗	✗
Thiols *(e.g. mercaptoethanol or DTT)*	✗	✗

*Please note that individually the concentrations shown should not affect the reaction. However in combination with additional compounds that are not recommended above a certain concentration, the reaction may be affected.

INSTRUCTIONS

1. Activation of Oligo

Recommended concentration and size of oligo for optimal results:

The kit can be used to conjugate both single and double stranded oligos. A single stranded oligo must be between 10 and 120 bases in length and contain a terminal amine group, which must be added during synthesis. All commercial oligo suppliers offer this modification. The efficiency of conjugation is slightly higher with 5'aminated oligos. Double stranded oligos are compatible up to 80 bases in length, but please note that only one end should be aminated.

The oligo must be HPLC purified, be 60-100µM concentration and be in at least 100µl of a suitable buffer. If the oligo concentration is greater than 100µM, dilute to 100µM in Wash Buffer. For conditions outside these recommendations, contact our Technical Support Team for advice.

Oligo Activation Procedure

Add 100 µl of the oligo into the Oligo Activation Reagent vial. Mix gently and incubate for 30 minutes at room temperature.
Meanwhile proceed to desalting procedure (step 3).

2. Activation of Antibody

Recommended antibody concentration and quantity of antibody for optimal results:

The Thunder-Link® oligo conjugation system kits are designed to activate 100 µg of antibody in 100 µl of a suitable buffer. The antibody to be activated must be purified and at a concentration of 1 mg/ml. Higher antibody concentrations should be diluted to 1 mg/ml with Wash Buffer. If your antibody is at a concentration of below 1 mg/ml, or is not in a suitable buffer (please see the section above regarding buffer considerations), please contact our Technical Support Team.

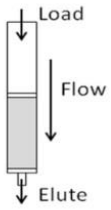
Antibody activation procedure

Add 100 µl of the 1 mg/ml antibody into the Antibody Activation Reagent vial. Mix gently and incubate for 30 minutes at room temperature.
Meanwhile proceed to desalting procedure (step 3).

3. Desalting Procedure

Use one column per Desalting. The columns are designed for single use. Discard after use.

1) Secure a separating column in a vertical position. Remove the two caps and allow the storage liquid to flow through the column to waste (remove the upper cap first).



2) Equilibrate column by adding 3ml of Wash Buffer to the top of the column and allow the liquid to flow through under gravity. Discard the flow-through. Repeat a further 4 times.
3) After the 30 minute incubation, add the 100µl of activated material (oligo or antibody) to the top of the column and allow the liquid to completely absorb into the column.
4) Add 550µl of Wash Buffer to the top of the column. This liquid is required to push the activated material to the base of the column. Allow this liquid to completely absorb before proceeding to the next step.
5) Place a collection vessel (not supplied) under the column. Add 300µl of Wash Buffer to the top of the column.
6) Collect the eluate from the column into the clean tube. This column eluate contains the activated material which is now free of Activation Reagent and ready to use.

STORAGE OF ACTIVATED MATERIAL

Oligo and control oligo

The activated oligo can be stored at room temperature for up to 8 hours. For longer storage of up to 12 months, -20°C is recommended.

Antibody and control antibody

The activated antibody should be stored on ice. It is very reactive and should be used within 2 hours. The activated antibody is not stable enough for long term storage.

GENERATION OF PURIFIED ANTIBODY / OLIGO CONJUGATE

This kit can be used to generate antibody:oligo conjugates with a range of different antibody:oligo ratios. This can be achieved simply by adding different amounts of oligo to the antibody as shown in the table below. The preferred ratio will depend upon your application, and may need to be determined experimentally (see optimisation instructions below).

Conjugation:

1) Add the 300µl of activated antibody to the appropriate volume of activated oligo and wash buffer as shown in the table below.

Volume of activated antibody(µl)	Volume of activated oligo (µl)	Volume of wash buffer (µl)	Antibody:oligo ratio*
300	300	0	1:15
300	200	100	1:10
300	100	200	1:5
300	60	240	1:3
300	20	280	1:1

*The antibody:oligo ratio is only ever an average since a population of labeled antibodies will be produced following the conjugation reaction. Each antibody will not have exactly the same number of oligos bound to it. When using a 1:1 ratio some unlabeled antibody may be present.

2) Mix and incubate at room temperature for 1 hour. Conjugations can also be incubated overnight at room temperature with no adverse effect.
3) Your conjugate is now ready for use. You may also purify the conjugate to remove any unbound oligo if this is required for your application (see conjugate purification information below).
4) Any unused activated oligo may be stored frozen.

OPTIMIZATION OF THE ANTIBODY: OLIGO RATIO

If you need to test which antibody:oligo ratio is best for your application this can be achieved by reducing the volume of antibody used and running several experiments using different ratios.

1) Mix together the volumes of antibody, oligo and wash buffer as shown in the table below.

Volume of activated antibody(µl)	Volume of activated oligo (µl)	Volume of wash buffer (µl)	Antibody:oligo ratio*
75	75	0	1:15
75	50	25	1:10
75	25	50	1:5
75	5	70	1:1

*The antibody:oligo ratio is only ever an average since a population of labeled antibodies will be produced following the conjugation reaction. Each antibody will not have exactly the same number of oligos bound to it. When using a 1:1 ratio some unlabeled antibody may be present.

2) Mix and incubate at room temperature for 1 hour. Conjugations can also be incubated overnight at room temperature with no adverse effect.
3) Your conjugates are now ready for use. You may also purify the conjugate to remove any unbound oligo if this is required for your application – see conjugate purification information below).
4) Any unused activated oligo may be stored frozen.

CONJUGATE PURIFICATION

Purification of 600µl of conjugate:

1) Warm the Conjugate Clean Up Reagent by placing the tube in warm water (not warmer than 40°C) for 10 minutes and mixing regularly. If the sample does not dissolve completely, spin the sample in a bench top micro-centrifuge at a recommended maximum speed of 13,000g for 1 minute, and use the supernatant.
2) Add 600µl of Conjugate Clean Up Reagent to the antibody/oligo mixture, mix and incubate at room temperature or on ice for 20 minutes.
3) Centrifuge in a bench top micro-centrifuge for 5 minutes at 15,000g. *Position the Eppendorf tube in the centrifuge in such a manner that you know where your pellet will be located.
4) Remove sample from the centrifuge taking care not to dislodge the small pellet at the bottom of the tube. If no pellet is seen add more Conjugate Clean Up Reagent (another 1/10 volume), mix well and incubate on ice for a further 10 minutes and centrifuge. If no pellet is seen when using a protein other than an antibody add another 600 µl of Conjugate Clean Up Reagent, mix well and incubate on ice for a further 10 minutes and centrifuge.
5) Carefully remove the supernatant and store until efficient precipitation has been confirmed.
6) Add 100µl of the Antibody Suspension Buffer to the pellet and mix gently.
7) The antibody/oligo conjugate is now ready to use.

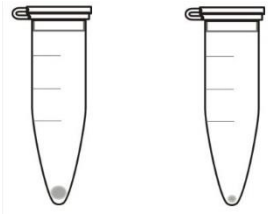
Purification of 150µl of conjugate:

Caution: As the amount of material being handled is very small this purification procedure is much more technically demanding due to the small size of the pellet that is formed. 50ug of antibody is the lower limit for seeing a clearly visible pellet.

1) Warm the Conjugate Clean Up Reagent by placing the tube in warm water (not warmer than 40°C) for 10 minutes and mixing regularly. Make sure any crystals have been re-dissolved, shake if necessary.
2) Add 150 µl of Conjugate Clean Up Reagent to the 150 µl of antibody/oligo mixture, mix and incubate at room temperature or on ice for 20 minutes.
3) Centrifuge in a bench top micro-centrifuge for 5 minutes at 15,000g. *Position the Eppendorf tube in the centrifuge in such a manner that you know where any pellet will be located.
4) Remove sample from the centrifuge taking care not to dislodge the very small pellet at the bottom of the tube. If no pellet is seen when using an antibody add more Conjugate Clean Up Reagent (another 1/10 volume), mix well and incubate on ice for a further 10 minutes and centrifuge. If no pellet is seen when using a protein other than an antibody add another 150 µl of Conjugate Clean Up Reagent, mix well and incubate on ice for a further 10 minutes and centrifuge.
5) Carefully remove the supernatant and store until efficient precipitation has been confirmed.
6) Add 50 µl of the Antibody Suspension Buffer to the pellet and mix gently.
7) The antibody/oligo conjugate is now ready to use.

*The required spin time will vary depending on buffer composition and speed. The speed should not exceed 15,000g.

Schematic to show pellet formation and approximate size



The image on the left shows the expected pellet size after incubation of 600ul 1mg/ml antibody with 600ul Conjugate Clean Up Reagent. The image on the right illustrates the expected pellet size following incubation of 150ul 1mg/ml antibody with 150ul Conjugate Clean Up Reagent. Eppendorf tubes are 1.5ml volume. 50ug of antibody is the lower limit for seeing a clearly visible pellet.

USE OF THE CONTROL OLIGO AND ANTIBODY (OPTIONAL)

Each conjugation kit is supplied with both a control oligo (a 30 base oligo with a 5' terminal amine) and a control antibody (rabbit IgG). These reagents are included as positive controls in order to give the option of confirming the conjugation chemistry is working optimally.

Procedure for activating the Control Oligo / antibody

1) Add 100µl of Wash Buffer to both the lyophilized vials of control oligo and control antibody.
2) Add the 100µl of Control Oligo to a vial of Oligo Activation Reagent. Mix and incubate at room temperature for 30 minutes.
3) Add the 100µl of control antibody to a vial of Antibody Activation Reagent. Mix and incubate at room temperature for 30 minutes.
4) Meanwhile proceed to the desalting procedure (step 3).

Note that you will require two columns. One column is required for the control oligo and a second column is for the control antibody.

GENERATION OF CONTROL ANTIBODY/OLIGO CONJUGATE

This kit can be used to generate a Control antibody/oligo conjugate with a range of different antibody:oligo ratios. This can be achieved simply by adding different amounts of oligo to the antibody as shown in the table below. The preferred ratio will be the one used to generate your own antibody/oligo conjugate.

Conjugation:

1) Add the 300 µl of activated antibody to the appropriate volume of activated oligo and wash buffer as shown in the table below.

Volume of activated antibody(µl)	Volume of activated oligo (µl)	Volume of wash buffer (µl)	Antibody:oligo ratio*
300	300	0	1:15
300	200	100	1:10
300	100	200	1:5
300	60	240	1:3
300	20	280	1:1

*The antibody:oligo ratio is only ever an average since a population of labeled antibodies will be produced following the conjugation reaction. Each antibody will not have exactly the same number of oligos bound to it. When using a 1:1 ratio some unlabeled antibody may be present.

2) Mix and incubate at room temperature for 1 hour. Conjugations can also be incubated overnight with no adverse effect.
3) Your conjugate is now ready for use. You may also purify the conjugate to remove any unbound oligo if this is required for your application (see conjugate purification information on page 3).
4) Any unused activated oligo may be stored frozen.

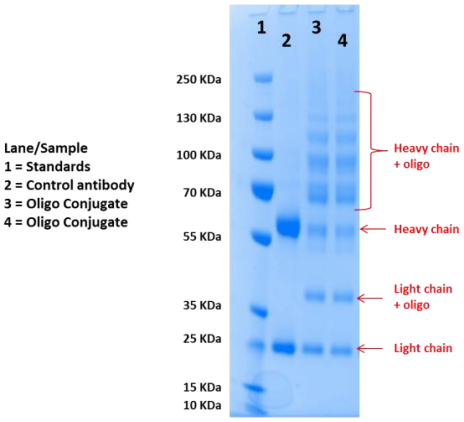
ANALYSIS OF THE ANTIBODY / OLIGO CONJUGATE

The generated conjugates can be analyzed in a variety of ways. The best method to confirm conjugation is a positive result in the chosen application. Alternatively, the conjugates can be analyzed using Gel Electrophoresis.

A small amount (5 to 10 µg) of the conjugate can be run on a reducing SDS/PAGE gel. The small sample of conjugate should be mixed with the 2X gel loading buffer (not supplied) and heated at 100°C for 5 minutes. This treatment will break all the disulphide bonds present in the antibody.

The sample should be allowed to cool before being loaded onto the SDS/PAGE gel (not supplied). A 4 to 12% gradient gel is recommended for best results. The gel is then run and stained for protein using Coomassie Blue stain or a suitable equivalent. After destaining the gel can be imaged to reveal the presence of antibody/oligo conjugates. A typical gel image for an IgG is shown below.

Reducing SDS-PAGE after Oligo Conjugation



Note: IgG consists of two heavy and two light chains. Not all of these chains will be attached to an oligo. There will be a number of unlabelled heavy and light antibody chains even within an excellent conjugate. This is especially true for low ratio conjugates.

Antibody chains attached to oligos may not stain as efficiently as unlabelled antibody chains. The gel images should therefore be considered as qualitative rather than quantitative.

The size of the shift in the heavy chain will depend on the size of the oligo conjugated. Larger oligos will generate a larger band shift and smaller oligos a smaller shift. The oligo used in the example is a 30mer. Other antibody subtypes, such as IgM will generate a different banding pattern on the gel.

TECHNICAL SUPPORT

For technical enquiries get in touch with our technical support team at: www.expedeon.com/contact/