Instruction for Use

Exazym® ClickChem Conjugation Kit 50

Catalogue Number 10-0001-01

For research use only. Not for use in diagnostic procedures.

This Instruction for Use (IFU) describes how to store, prepare and correctly use Exazym® ClickChem Conjugation Kit 50 (cat no. 10-0001-01). The resulting oligo-dT primer conjugated detector antibody may then be used to implement the BOLD technology using Exazym® Polymerase Reaction Kit and Exazym® Biotin Detection Kit for signal amplification of immunoassays.

Introduction to BOLD

Measurement of low abundance biomolecules remains a challenge in many pre-clinical, clinical and diagnostic applications due to insufficient sensitivity. While some biomarker discovery and detection as well as clinical diagnostic measurement methods have made significant advances in sensitivity, there are still many potential disease biomarkers that exist in accessible biofluids at levels below the detection limits of these techniques or where an increased precision is desirable. Furthermore, they require specialized instruments, increasing the cost and logistical complexity of large-scale adoption.

Cavidi has developed a range of Exazym® reagents and kits based on its proprietary BOLD signal amplification technology. BOLD brings ultra-sensitive detection levels to conventional immunodiagnostic assays and "BOLD" stands for Binding Oligo Ladder Detection. When using BOLD an oligo-dT primer conjugated to a detector antibody is mixed with a polymerase containing reverse transcriptase activity, 5-Bromo-2'-deoxyuridine 5'-triphosphate monomers (BrdUTP) and a rA-template to create a long hybrid-ladder of DNA:RNA to which biotinylated anti-BrdU detection antibodies selectively bind to enable the amplified signal. Integration of BOLD into an immunoassay improves the sensitivity and the limit of detection can be improved by up to 50x.

Signal enhancement using BOLD and Exazym® Kit System is useful for signal enhancement of immunoassays in particularly in translational research, health screening and diagnostics testing. Examples of such applications are biomarker discovery and detection, especially in the field of low abundance proteins, *in vitro* diagnostics in the fields where improved detection levels are crucial such as neurology, cardiology, and monitoring of relapse in already treated cancer patients.

What is Exazym® ClickChem Conjugation Kit 50?

Exazym® Kit System consists of three products necessary to perform BOLD; i) conjugation of a primer to the detector antibody, ii) the polymerization phase to generate the hybrid ladder of DNA:RNA and, iii) binding of the biotinylated anti-BrdU detection antibodies to the hybrid ladder of DNA:RNA to provide the final signal. All required components for these three unique events of BOLD are included in:

- Exazym® ClickChem Conjugation Kit 50 and 250 - for conjugation of Exazym® primer to the detector antibody of choice.
- Exazym® Polymerase Reaction Kit 96 and 960 - for performing the polymerase reaction when applying BOLD technology for signal amplification of immunoassays.
- Exazym® Biotin Detection Kit 96 and 960 - for detection of the DNA:RNA hybrid by a biotinylated anti-BrdU antibody; Exazym® Antibody Biotin.

Exazym® ClickChem Conjugation Kit 50 is intended for conjugation of an oligo-dT primer to 50 µg of detector antibody using Click Chemistry. The kit is intended for research use only and not for diagnostic procedures.

Product specification

The reagents required for conjugation of oligo-dT primer to 50 µg of a detector antibody using Click Chemistry. Exazym® ClickChem Conjugation Kit 50 is designed to be used together with Exazym® Polymerase Reaction Kit 96 or 960 and Exazym® Biotin Detection Kit 96 or 960 and can bring ultra-sensitive detection levels to conventional immunodiagnostic assays.

Components included in the kit

Components in Pack 1:

1 vial 3 mL DMSO, anhydrous. The vial is for single use only and any remaining DMSO should be discarded.

2 Spin Columns

1 tablet PBS-T to be reconstituted in 100 mL de-ionized water.

Components in Pack 2:

1 vial 75 µg lyophilised Exazym® Oligo-dT Primer (DBCO-TEG-30-mer), (9 634 g/mol)

1 vial 5 mg evaporated ClickChem Label (NHS-PEG4-azide), (388.37 g/mol)

1 vial 1 mL, 1 M Quenching Buffer

Materials and equipment required but not included

2.0 mL Eppendorf vials

2.0 mL collection vial (Eppendorf vial with removed cap)

De-ionized water

Micro-centrifuge ≥700 RCF

Micro BCA Protein Assay Kit (cat no #23235), ThermoFisher may be used for quantification of conjugated antibody.

SDS-polyacrylamide gel electrophoresis may be used to visualize the conjugation.

Pipettes and other standard laboratory equipment

Shipping conditions

Shipped on wet ice.

Storage conditions

Pack 1 is stored at +15 °C to +30 °C. Prepared PBS-T solution is stored at +2°C to +8 °C.

Pack 2 is stored at -30 °C to -15 °C.

Warnings

This is a single-use kit. Please follow the instructions below and discard any used and old vials or chemicals. Do not re-use any vials or chemicals.

Do not use a kit which is broken upon arrival. Please contact customer support immediately (see below for contact details).

This product is for research and laboratory use only and shall be handled by professional and trained users only.

Personal protective equipment shall be used such as eye shield and gloves when handling the kit.

For further safety information, please refer to the respective Material Safety Data Sheet on Cavidi's web page or call customer support (see below for contact details).

How to use Exazym® ClickChem Conjugation Kit 50

Preparation of detector antibody prior to use in case of presence of azide or primary amines

Note: This step is only necessary if the detector antibody solution contains azide or components that contain primary amines, (e.g. Tris and glycine). These will interfere with the conjugation reaction of the ClickChem Label.

You may use one of the spin columns supplied with the kit to remove the components containing primary amines from the solution before conjugation.

Make sure that the concentration of the initial antibody solution is high enough to obtain a solution after the spin column procedure with a concentration of at least 1 mg/mL, below (see step 9). An optimal concentration of the initial antibody solution is ≥1.5 mg/mL.

Procedure:

1. Remove the bottom closure of the spin column. Loosen the cap (do not remove cap). **Note:** all centrifugation steps must be performed with a loosened cap.

2. Using a lab marker pen, make a vertical line on the outside of the spin column. Ensure that the line is faced outward (away from the centre of the rotor) in all centrifugation steps.

3. Place the spin column into a 2 mL collection vial and spin the column in the micro-centrifuge at 700 x g (700 RCF) for 1 min to remove the storage solution.

4. Discard the flow-through and place the spin column into the collection vial again.

5. Add 300 µL PBS-T buffer on top of the resin. Centrifuge spin column placed in the collection vial at 700 x g (700 RCF) for 1 min and discard flow-through. Repeat this step twice. During the last (3rd washing step) centrifuge the spin column at 700 x g for 2 min.

   After each centrifugation, the resin should appear white and free of liquid. If liquid is present, ensure that you have used the correct centrifugation speed and time. Incomplete centrifugation can result in poor recovery or dilution of the sample.

6. Using for example, a clean filter paper, blot the bottom of the column to remove excess liquid. This is to avoid unnecessary dilution of the sample.

7. Transfer the column to a new collection vial.

8. Apply antibody solution on top of the resin. If the sample volume is < 70 µL, add a volume of PBS-T, to obtain a total sample volume of 70 µL, as soon as the sample has entered the resin.

9. Centrifuge tube at 700 x g (700 RCF) for 2 min and retain the flow-through that contains the sample. Discard the spin column.

10. Before conjugating the prepared detector antibody, we recommend that you measure the concentration of the antibody sample and as needed, adjust the concentration to 1.0 mg/mL using PBS-T. This concentration will ensure that you can start the conjugation procedure using 50 µg of antibody in a volume of 50 µL.

Preparation of ClickChem Label prior to use

1. To avoid moisture in the vials, allow the freeze-dried ClickChem Label to reach room temperature before use.

2. Centrifuge the vial containing the ClickChem Label at maximum speed for 1 min to collect the ClickChem Label at the bottom of the vial.

3. To prepare a 100 mM ClickChem Label stock solution, reconstitute the freeze-dried ClickChem Label by adding 128.5 µL anhydrous DMSO to the vial. Gently vortex the solution using medium speed for 10 seconds to assist reconstitution. Allow the pellet to rehydrate for 1 min. Then gently vortex the solution for 10 seconds a second time. The 100 mM Click Chem Label stock solution is stored at -30 to -15 °C.

4. Immediately before use, prepare a 0.5 mM ClickChem Label solution by adding 5 µL of 100 mM ClickChem Label stock solution to 995 µL PBS-T.

5. Use the 0.5 mM solution to add the desired molar excess to the antibody in the conjugation reaction. Recommended molar excess of ClickChem Label versus non-conjugated detector antibody is 5x-20x. For examples of calculations for 5x and 20x excess, please refer to Table 1.

Preparation of Exazym® oligo-dT Primer prior to use

1. To avoid moisture in vials, allow the freeze-dried Exazym® oligo-dT Primer to reach room temperature before use.

2. Centrifuge the vial containing lyophilized Exazym® Oligo-dT Primer at maximum speed for 1 min to collect the lyophilized primer at the bottom of the vial.

3. To prepare a 210 µM solution, add 37 µL PBS-T to the vial with freeze-dried Exazym® oligo-dT Primer.

4. Mix gently for 10 seconds twice using a Vortex at medium speed. After each mixing, spin down the solution and allow the pellet to re-hydrate for 1 min. Store primer dissolved in PBS-T at -30 to -15 °C.

6. Use the 210 µM solution to add molar excess to the ClickChem labelled antibody. Recommended molar excess of Exazym® Oligo-dT Primer to non-conjugated antibody is 5x-20x. For examples of calculations for 5x and 20x excess, please refer to Table 1.

Note: Molar excess of ClickChem Label and Exazym® oligo-dT Primer may be optimised for each antibody to be conjugated by testing other combinations of the two components using 5x-20x molar excess. See calculations in Table 1 for examples of 5x and 20x molar excess.

Concentration of antibody solution	Mw (g/mol)	Conc. (mg/mL)	Conc. (µM)	Vol. (µL)	nmol/50 µL
1.0 mg/mL	150 000	1.0	6.7	50.0	0.33

Molar ratio of ClickChem Label vs antibody	Mw (g/mol)	Conc. (mM)	nmol ClickChem Label to be added to antibody	Volume (µL) ClickChem Label solution to be added to antibody	Volume (µL) of antibody already added	Total volume (µL) ClickChem Label and antibody in vial	Volume (µL) of Quenching Buffer to be added to 75 mM in 100 µL	Final volume (µL) of PBS-T to be added to reach a final vol of 100 µL
5x	388.4	0.5	5 x 0.33	3.3	50.0	53.3	7.5	39.2
20x	388.4	0.5	20 x 0.33	13.3	50.0	63.3	7.5	29.2

Molar ratio of Exazym® oligo-dT Primer vs ClickChem labelled antibody	Mw (g/mol) Exazym® Oligo-dT Primer	Quantity (µg) Exazym® Oligo-dT Primer	Quantity (nmol)	Volume (µL) of PBS-T to be added to 75 µg Exazym® Oligo-dT Primer	Volume (µL) of Exazym® Oligo-dT Primer to be added to ClickChem labelled antibody
5X	9 634	75	7.8	37	8
20X	9 634	75	7.8	37	32

Table 1. Example of reaction volumes for 50 µg antibody conjugation adding 5x and 20x molar excess of Exazym® ClickChem Label and 5x and 20x excess of Exazym® oligo-dT Primer. Volumes of Quenching Buffer and PBS-T to be added to the ClickChem labelled detector antibody mixture as well as volume of PBS-T to be added to freeze-dried Exazym Oligo-dT Primer are also included in the table.

Procedure for preparation of ClickChem labelled detector antibodies

1. In a 2 mL Eppendorf vial, add 50 µg of the detector antibody. **Note:** Please refer to section "Preparation of detector antibody prior to use in case of presence of azide or primary amines" above in case your detector antibody solution contains azide or primary amines.

2. Using the prepared 0.5 mM ClickChem Label solution, add 5x or 20x molar excess of label versus the amount of detector antibody. Please refer to the calculations in Table 1 for examples of 5x and 20x molar excess.

3. Incubate vials at room temperature for 30 min.

4. Stop the labelling reaction by adding Quenching Buffer (1 M) to a final concentration of 75 mM and add PBS-T to a total volume of 100 µL according to the calculations in Table 1.

5. Mix gently and incubate the quenched reactions for 5 min at room temperature.

6. To remove any uncoupled/deactivated label use the spin column provided in the kit. Remove the spin column's bottom closure. Loosen the cap (do not remove cap). **Note:** all centrifugation steps must be performed with a loosened cap.

7. Using a lab marker pen, make a vertical line on the outside of the spin column. Ensure that the line is faced outward (away from the centre of the rotor) in all centrifugation steps.

8. Place the spin column into a 2 mL collection vial and centrifuge at 700 x g (700 RCF) for 1 min to remove the storage solution.

9. Discard flow-through and put the spin column into the collection vial again.

10. Add 300 µL PBS-T buffer on top of the resin. Centrifuge spin column placed in the collection vial at 700 x g (700 RCF) for 1 min and discard flow-through. Repeat this step twice. During the last (3rd washing step) centrifuge the spin column at 700 x g for 2 min.

11. After each centrifugation, the resin should appear white and free of liquid. If liquid is present, ensure that you have used the correct centrifugation speed and time. Incomplete centrifugation can result in poor recovery or dilution of the sample.

12. Using for example, a clean filter paper, blot the bottom of the column to remove excess liquid. This is to avoid unnecessary dilution of the sample.

13. Transfer the column to a new collection vial. Add the ClickChem labelled antibody from step 5 on top of the resin (100 µL).

14. Centrifuge for 2 min at 700 x g (700 RCF) and retain the flow-through that contains the ClickChem labelled detector antibody. Discard the spin column.

Conjugation of Exazym® Oligo- dT Primer to ClickChem labelled antibodies

1. Add Exazym® oligo-dT Primer (210 µM) to the ClickChem labelled antibody. A molar excess of 5x-20x of the oligo-dT Primer versus the ClickChem labelled antibody is recommended. Refer to Table 1 for calculations describing a 5x and 20x molar excess of Primer to 50 µg of ClickChem labelled antibody.

2. Incubate Exazym® oligo-dT Primer with ClickChem labelled antibody for 16-24 hours in a dark environment and at room temperature. Store the conjugated antibody at 2 to 8 °C.

Note: Quantification of conjugated antibody may be done using Micro BCA Protein Assay Kit (cat no #23235) from ThermoFisher and interpolate measured samples with a standard curve using Bovine IgG dilutions. Conjugation success can be visualized by running a reduced SDS-PAGE gel (appr. 5 µg antibody per lane).

Intellectual Property rights

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How do I get in contact with Cavidi's tech support?

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