Protocol Booklet

Product Code(s)	HB9768 / HB9660
Product Name(s)	Janelia Fluor® 525 conjugation kit Janelia Fluor® 549 conjugation kit
Purpose	Conjugation of proteins to Janelia Fluor® dyes

Please note: This product is for RESEARCH USE ONLY and is not intended for therapeutic or diagnostic use. Not for human or veterinary use

Contents

Product Overview

Hello Bio Janelia Fluor® conjugation kits allow the conjugation of antibodies and proteins to Janelia Fluor® dyes in as little as 90 minutes (15 minutes active time) with a high degree of labelling. There are many benefits of directly labelled proteins and antibodies such as:

- Much easier multiplexing - no need to mix and match antibody species correctly,
- Avoid non-specific binding by secondary antibodies,
- Save time by shortening staining protocols.

To have the best chance of successfully labelling the protein or antibody and achieve the optimal degree of labelling we recommend:

- Ensure the target protein does not contain BSA or other stabilising proteins as this will reduce the target labelling. These should be removed before processing.
- The protein to be labelled should be greater than 7kDa in size.
- The protein concentration should be at least 1mg/ml, lower concentrations can be used however this will influence the degree of labelling.

Components & Storage

This kit contains:

- Lyophilised Janelia Fluor dye
- DMSO
- Lyophilised conjugation buffer
- Lyophilised quenching buffer
- Protein storage buffer
- Microfuge desalting columns

Note: Store all kit components at 4°C until use except from the lyophilised Janelia Fluor dye which should be stored at -20°C

This kit additionally requires:

- Benchtop microcentrifuge capable of accepting 1.5ml sized microcentrifuge tubes
- 1.5ml microcentrifuge tubes
- Rockers or wheel at room temperature

Protocol

2.1 Preparation

Before initiating a conjugation reaction there are a few key steps that need to be carried out:

1. Calculate the required volumes of protein, Janelia Fluor® dye, conjugation buffer, quenching buffer and storage buffer. Please note that the minimum volume of the spin column is 30µl so any samples of lower volume than this will need to be diluted.

    a. A good starting point is using a 15:1 molar ratio of dye : protein.
    b. The volume of conjugation buffer needs to be sufficient for 3 x 300µl column washes per reaction.
    c. The volume of quenching buffer should be 10% of volume of the total reaction mixture (Janelia Fluor dye and protein in conjugation buffer).
    d. The volume of storage buffer needs to be sufficient for 3 x 300µl column washes per reaction in addition to any required to dilute the resulting labelled protein.

2. Prepare the reagents by:

    a. Reconstituting the lyophilised conjugation buffer with 5ml of dH_2O. Surplus buffer can be frozen at -20°C and used in future conjugation reactions.
    b. Reconstitute the lyophilised quenching buffer with 250µl of dH_2O. Surplus buffer can be frozen at -20°C and used in future conjugation reactions.
    c. Reconstitute the lyophilised Janelia Fluor® with the volume of anhydrous DMSO calculated. Please note that the Janelia Fluor® dye is unstable once reconstituted therefore should be used within 24 hours.

3. Prepare your workspace and ensure that all reagents are prepared and ready to use.

2.2 Preparing the Sample

1. Remove the bottom of a spin column, loosen cap and place into a microfuge tube and spin at 1,500g for 1 minute to remove storage buffer. Discard the contents of the microfuge tube. Mark the side of the column where the resin has been pushed upwards and make sure that this mark is facing outwards at all future centrifugation steps.

2. Add 300µl carbonate buffer to the column and spin at 1,500g for 1 minute. Discard the flowthrough. Repeat twice for a total of 3 washes.

3. Change the collection tube, remove the cap fully and apply protein to the top of the bed, allowing it to fully absorb.
    a. 30-130µl can be run through the column at a time. If the volume of protein is under 70µl then apply a 15µl stacker of dH_2O.

4. Centrifuge at 1,500g for 2 minutes and **keep the flowthrough**, this is the protein in conjugation buffer.

2.3 Conjugation

1. Add the Janelia Fluor dye to the protein solution, incubate at room temperature in the dark for 60 minutes with gentle agitation.

2. Add quenching buffer and incubate in the dark for 10 minutes at room temperature with gentle agitation.

2.4 Cleaning up the Sample

1. Remove the bottom of a spin column, loosen cap and place into a microfuge tube and spin at 1,500g for 1 minute to remove storage buffer. Discard the contents of the microfuge tube. Mark the side of the column where the resin has been pushed upwards and make sure that this mark is facing outwards at all future centrifugation steps.

2. Add 300µl protein storage buffer to the column and spin at 1,500g for 1 minute. Discard the flowthrough. Repeat twice for a total of 3 washes.

3. Change the collection tube, remove the cap fully and apply conjugation mixture to the top of the bed, allowing it to fully absorb.
    a. 30-130µl can be run through the column at a time. If the volume of protein is under 70µl then apply a 15µl stacker of dH_2O.

4. Centrifuge at 1,500g for 2 minutes and **keep the flowthrough**, this is the conjugated protein in storage buffer.

5. Store the purified Janelia Fluor conjugated protein at 4°C away from light. This will be stable for up to 6 months at 4°C and indefinitely if snap frozen then stored at -80°C. Avoid freeze thaw cycles.

2.4 Calculating Degree of Labelling

1. Dilute a small amount of the conjugated protein into PBS and measure the absorbance at 280nm (A_{280}) and the maximum absorbance wavelength of the Janelia Fluor dye (A_{max}) in a cuvette.

    a. Calculate the concentration of the protein in the sample with the equation:

$$\text{Protein concentration (M)} = \frac{\left(A_{280} - \left(A_{dye} \times CF_{280}\right)\right) \times \text{dilution factor}}{\text{Protein extinction coefficient } (\varepsilon)}$$

    The protein extinction coefficient (ε) differs between proteins with a standard value for IgG being 203,000 $cm^{-1}M^{-1}$at 280nm. If you do not know the ε of the protein then there are tools that allow its estimation.

    b. The dilution factor is the ratio of the diluted protein to the stock solution (e.g. 1 in 100 dilution would have a dilution factor of 100)

    c. These calculations are based upon the assumption of a 1cm pathlength. If using a different cuvette (or nanodrop) then multiply the ε of both the protein and dye by the correction factor given by this equation:

$$\text{Pathlength correction factor} = \frac{10}{\text{Pathlength(mm)}}$$

2. For the CF_{280} please find the details in the below table:

Catalogue Number	Dye	Molecular Weight (g/mol)	Maximal absorbance (nm)	Maximal emission (nm)	ε_{dye} ($M^{-1}cm^{-1}$)	CF_{280}
-	Janelia Fluor 503	388.26	503	529	95,000	-
HB8455	Janelia Fluor 525	623.51	525	549	122,000	0.185
HB7336	Janelia Fluor 549	551.5	549	571	101,000	0.169
-	Janelia Fluor 585	649.6	585	609	156,000	0.099
-	Janelia Fluor 635	629.69	635	652	167,000	0.0524
-	Janelia Fluor 646	618.75	646	664	152,000	0.19
-	Janelia Fluor 669	693.77	669	682	116,000	0.043

4. Calculate the degree of labelling with the equation:

$$\text{Degree of labelling (Mol dye: Mol protein)} = \frac{A_{dye} \times \text{dilution factor}}{\varepsilon_{dye} \times \text{protein concentration (M)}}$$

    i. The degree of labelling should be around 1-10 mol of dye : mol protein.

Guidelines, precautions, troubleshooting

Please follow the below table to resolve any problems encountered when using this kit. For any problems not listed or for any further advice please contact our technical support team at technicalhelp@hellobio.com.

Problem	Potential Cause
Poor labelling either determined through degree of labelling calculations or experimentally.	Trace amine contamination in protein buffer inhibiting labelling. The labelling reaction is inhibited by primary amines that may not all have been removed during the sample preparation stage. Extensively dialyse the protein against an amine-free buffer such as PBS before re-attempting labelling.
	Protein concentration too dilute. Protein concentrations lower than 1mg/ml will not label as well as higher concentrations.
	Protein variability. Some proteins label more effectively than others therefore will need different molar ratios or incubation conditions. Try increasing the molar ratio above 15 alongside increasing the length of incubation during the labelling step.
Over labelling either determined through degree of labelling calculations or observation of either protein aggregation or loss of antibody specificity	Over labelling may impair the function of the labelled protein or antibody, causing impaired function or target recognition. Try reducing the molar ratio of dye:protein during the labelling.
Inefficient removal of free dye	It is possible that for some Janelia Fluor dyes that some trace dye manages to bypass the sample clean up stage which will give erroneously high degree of labelling values. To correct this run the sample through another spin column or use dialysis.

Observe safe laboratory practice and consult the safety datasheet. Please see the datasheet on our website for general guidelines, precautions, limitations on the use of the assay kit.

Contact

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