INFO SHEET

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Cell Line Development (CLD)

Detection Reagents for the Custom Productivity Assay

Including the Bispecific Heterodimer Assay, on Beacon® Optofluidic Systems

INTRODUCTION

Detection reagents for the Custom Productivity and Bispecific Heterodimer Assays are fluorescent molecules that bind to the secreted protein of interest and are used to quantify the amount of this protein produced by the cells being assayed. There are many approaches to producing reagents that are suitable for use in the assay, but in many cases, the detection reagent will be a protein, such as an antigen, that has been labeled with a fluorophore. This info sheet outlines considerations for using such labeled proteins as detection reagents. These recommendations are not intended to provide an exhaustive review of all possibilities and may not address the specific needs of any given project. Regardless of how a detection reagent is produced, it must meet the following requirements:

- The detection reagent should bind the desired target with high specificity, with a KD less than 50 nM.
- A 4-chip workflow with 1 assay will require ~6.5 nanomoles of detection reagent. More reagent may be required per assay depending on the molecular weight of the detection reagent and the secretion rate of the cells.
- The detection reagent must be soluble at the working concentration.
- The molecular weight of the detection reagent should be less than 150 kDa.
- The detection reagent needs to be animal component free to be compatible with FDA guidelines and should not contain any toxic additives.
- For characterization of bispecific molecules using the Bispecific Heterodimer Assay, a detection reagent valency of 1 is required.
- A degree of labeling (DOL) of at least one dye molecule per detection molecule is recommended. A lower DOL may be acceptable provided there is adequate fluorescence signal.

PROTEIN LABELING

We have had success with Thermo Fisher Scientific's **Amine-Reactive Probes** and recommend following the associated protocols and guidance, provided there are no case-specific reasons to choose other options. If amine-reactive labels are not suitable for your project, suppliers of fluorescent probes have a variety of other conjugation chemistries available. For instance, Thermo Fisher Scientific's **Molecular Probes Handbook** has information on a variety of labeling options that they offer.

NOTE: *BSA and sodium azide should be avoided as reagents used at any point in the labeling process.*

Determining the Amount of Labeled Protein Required for the Beacon® Optofluidic System Workflow

The amount of unlabeled protein, in nanomoles, needed for the labeling reaction is presented in **TABLE 1**. These values assume an expected recovery of 40% and include 10% overage.

TABLE 1: Amount of unlabeled protein (nanomoles) required

# Assays*	1 Chip	2 Chips	3 Chips	4 Chips
2	4.47	8.94	13.41	17.88
3	6.70	13.41	20.11	26.81
4	8.94	17.88	26.81	35.75

** 1 of the assays listed here will be used for reference images.*

Purchasing Reagents

All materials that are used to produce labeled proteins for detection must be animal component free and should not contain any toxic additives to comply with FDA regulations. **TABLE 2** presents some recommendations for products and suppliers. Ensure that proteins are dissolved in buffers that are compatible with the labeling reaction. For example, avoid buffers that include amines or detergents when using amine-reactive probes.

TABLE 2: Purchased reagent recommendations

Reagent	Supplier	Link	Advantages
Alexa Fluor® 594	Thermo Fisher Scientific	A37572	• Certificates of Origin provided • Optimal for the TRED channel on the Beacon system • High quantum yield and photostability • Good water solubility • High purity
Alexa Fluor® 647	Thermo Fisher Scientific	A20006	• Certificates of Origin provided • Optimal for the CY5 channel on the Beacon system • Low background • Low absorbance at 280 nm • High quantum yield and photostability • Good water solubility • High purity
Proteins	ACROBiosystems	ACRO products	• Certificates of Origin provided • A comprehensive list of unlabeled proteins is available • High quality • Custom proteins • Labeled proteins may be available

Third-party Labeling Services

An alternative to labeling a protein in-house is to use a third-party service. If a labeling service is used, ensure that their process does not include animal-derived components (such as BSA) or toxic compounds (like sodium azide, a common preservative).

The detection reagent concentration is provided during the workflow set up, and an accurate concentration and degree of labeling is highly recommended to ensure the best workflow performance. Either ensure that the labeling service can provide accurate measurements or confirm the protein concentration independently.

Performing the Labeling Reaction

Carefully review any protocols or documentation from the supplier of the fluorescent label you are using to ensure the protocol meets your needs and to budget the appropriate time and resources.

A degree of labeling of at least one dye molecule per protein molecule is recommended. A high degree of labeling may increase the risk that the label interferes with the binding of the detection reagent to the secreted bispecific molecule. Some experimentation and adjustment of labeling parameters may be necessary to achieve the proper degree of labeling and to ensure that binding is not impaired.

Removing Unreacted Dye

Free fluorophore must be separated from the labeled protein before quantitation and use in the assay. Multiple methods exist for this separation. We recommend following any guidance provided with the fluorescent probe conjugation protocol you are using. It may be necessary to perform multiple rounds of separation to fully remove free dye. By the end of the purification, there should be no color remaining in the fraction that would contain the unreacted dye. This indicates that all the fluorophore that can be separated from the protein has been removed.

After labeling and purifying the protein, filter the sample through a 0.2 µm filter using a sterile syringe.

Quantitation of Detection Reagent

To ensure an appropriate amount of reagent is used, the concentration and degree of labeling of the detection reagent should be measured prior to performing the assay. Both concentration and degree of labeling can be determined by UV-visible absorbance spectroscopy.

The degree of labeling is the average amount of conjugated dye per protein, which can be calculated by the ratio of the concentration of dye to protein. The concentration of dye can be determined by absorbance at its absorbance maximum. The concentration of most proteins can be determined by absorbance at 280 nm. However, since many fluorescent dyes absorb at 280 nm as well, a correction factor needs to be applied for the absorbance attributed to the dye. Refer to the section "Determining the correction factor for a dye" below.

Thermo Fisher Scientific provides a detailed protocol for the determination of the degree of labeling, found at **Calculate dye:protein (F/P) molar ratios**.

As the measured concentration is directly proportional to the extinction coefficient, any error in the extinction coefficient will be propagated directly to the concentration measurement. Consequently, it is important that extinction coefficients be as accurate as possible.

TABLE 3: Extinction coefficients for recommended dyes

Dye	Extinction Coefficient (at absorbance maximum)
Alexa Fluor® 594	92,000 $M^{-1}cm^{-1}$
Alexa Fluor® 647	270,000 $M^{-1}cm^{-1}$

If an empirically determined extinction coefficient for the protein is not available, the extinction coefficient of an unlabeled protein can be calculated from the amino acid sequence, use **Expasy's ProtParam** tool. This calculation is based on the method described by Pace *et al.*, 1995. For a detailed explanation, refer to that paper, found here: **How to measure and predict the molar absorption coefficient of a protein**.

Achieving the necessary level of accuracy in absorbance measurements requires care. Here are some recommendations for taking absorbance measurements:

- Observe all best practices provided by the manufacturer of your spectrophotometer.
- Maintain and calibrate your spectrophotometer according to the manufacturer's guidelines.
- Ensure that the absorbance measurement is within the optimal range for your spectrophotometer. Values that are too low or too high may be unreliable.
  - If the absorbance value is too high, a dilution can be made to obtain a measurement within range. In this case, ensure that the instruments used to make the dilution, such as micropipetters, are properly maintained, within calibration, and used according to best practices.
- For fluorescently labeled proteins, ensure that you are properly correcting for absorbance at 280 nm caused by the fluorophore.
- Ensure that your protein sample is free from other species that absorb at 280 nm, such as other proteins, DNA, or buffer components.
- Ensure that your sample is free of particulates. This can be accomplished by filtering the sample prior to measurement.
- Ensure that the extinction coefficient is correct.
- When taking a blank measurement, use a solution that is as similar as possible to the solution in which your protein is dissolved.
- When applying the Beer-Lambert Law, make sure that you are using the correct path length.
  - Be aware that some instruments may report an absorbance reading that has been normalized to a path length of 1 cm, rather than the actual path length used for the measurement. If you are using such a normalized absorbance reading, use 1 cm as the path length for calculation.

Determining the Correction Factor for a Dye

Due to lot-to-lot variation in labels, we suggest that you determine the correction factor for the absorbance at 280 nm empirically. For this, you will need a sample containing the free dye in the same buffer in which your labeled protein will be measured at a concentration appropriate for measurement of the absorbance.

1. Measure the absorbance of the dye sample at 280 nm (A280) and at the dye's peak max (A_{max})
2. The correction factor is calculated as $CF=A_{280}/A_{max}$

This method assumes that changes to the absorbance spectrum upon conjugation to the protein are negligible. This is often a reasonable assumption but may not hold in all situations.

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