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Assessment of Free Dye in Solutions of Dual-Labeled Antibody Conjugates for *In Vivo* Molecular Imaging

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Abstract

PURPOSE—Recent preclinical and clinical studies show dyes that excite and fluoresce in the near infrared range may be used for tracking and detecting disease targets *in vivo*. A method for quantifying free dye molecules in antibody conjugate preparations is required for agent batch release and for translation into the clinic.

PROCEDURES—Herein, we developed and validated a SDS-PAGE method to determine the percentage of free IRDye 800CW in (DTPA)_n-trastuzumab—(IRDye 800)_m conjugate sample preparations in which HPLC assessment of free dye was not possible.

RESULTS—The SDS-PAGE assay was accurate and valid for free IRDye 800CW amounts between 38 and 4 molar percent of total dye. Gel sample preparation reagent affected the specificity of the assay, and lower and upper limits of quantitation and detection were determined.

CONCLUSION—This method may be applicable to other near infrared dye-conjugated antibody-based imaging agents in which HPLC assessment of purity is not feasible. This validated method for quality assurance will facilitate the translation of dual-labeled antibody conjugates for nuclear and optical imaging.

Keywords

NIR (near infrared); DTPA (diethylenetriaminepentaacetic acid); HPLC (high pressure liquid chromatography); TLC (thin layer chromatography); SEC (size exclusion chromatography); IRDye 800CW; CV (coefficient of variation)

1. Introduction

While nuclear imaging offers exquisite sensitivity that enables molecular imaging of disease targets for diagnosis and assessing response to therapy, near-infrared (NIR) fluorescence has also shown promising sensitivity for clinical application with microdosage [1, 2]. Unlike nuclear medicine techniques, however, there are no established and validated methods for assessing the purity of NIR-conjugated agents, such as fluorophores that have a reactive NHS (*N*-hydroxysuccinimide) ester for lysine binding on antibodies, antibody fragments, or

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other proteins. As a comparative example, prior to administration, the radiopurity of a radiopharmaceutical is evaluated, usually by thin-layer chromatography (TLC), to assess free isotope *versus* chelated isotope [3]. Since free, unbound radionuclide can distort or inhibit accurate imaging of the desired target, determination of the percent bound radionuclide is an important parameter to check before administering the agent for imaging. Acceptably low ratios can then be identified to minimize the background for nuclear imaging of *in vivo* targets.

A similar situation exists for NIR fluorescence, as free dye can create a significant background that can limit the performance of the molecular imaging approach. As with a radiolabeled conjugate, before a fluorescent agent can be administered *in vivo*, its fluorescence purity needs to be evaluated. Unfortunately, in the case of large proteins, such as antibodies or antibody fragments, the use of size exclusion high pressure liquid chromatography (HPLC) for assessing purity can be challenging, due to overlapping retention times of the fluorophore and chelators. In addition, unlike radioisotopes, the opportunity for quenching can distort quantification of free and bound fluorophore. In our work to validate “first-in-humans” NIR fluorescent agents, we focused on evaluating dual-labeled trastuzumab by developing and validating an analytical approach to assess the purity of the fluorescently labeled compound.

Trastuzumab (Herceptin®, Genentech, South San Francisco, CA) is a humanized antibody specific for the HER2/neu receptor overexpressed on some cancer cells, and HER2/neu has been used as a marker of certain aggressive breast, ovarian, and stomach cancers [4–13].

Radioimmunoscinigraphy studies have employed trastuzumab as well as other therapeutic antibodies [14–28] because of their high affinity for disease targets, but the short half-life of radionuclides and long circulation times may be responsible for their lack of universal adoption. Recently, we have demonstrated the ability to noninvasively detect lymph nodes following microdosage (i.e., 100 ug) of indocyanine green, a weak NIR fluorophore without a functional group for conjugation, suggesting the potential of using NIR fluorophores to label antibodies for high-affinity molecular imaging without concern of isotope half-life [29–32]. In order to provide a comparative assessment of the performance of NIR fluorescence with nuclear imaging, we have created a dual-labeled trastuzumab that uses the conjugated chelator DTPA (diethylene triamine pentaacetic acid) for sequestering ¹¹¹indium and the NIR fluorophore, (IRDye 800) to provide the optical signal [32]. The combination of both nuclear and fluorescent reporters offers unique opportunities for non-invasive nuclear imaging with subsequent fluorescence image-guided resection without concern of fluorophore half-life.

In this study, we developed and validated a method to ascertain quantities of molar percentages of free, or unbound, IRDye 800CW in preparations of (DTPA)_n-trastuzumab—(IRDye 800)_m. We examined common validation parameters, such as linearity, detection and quantitation limits, selectivity, precision, accuracy, and range in developing and validating this method [33, 34]. This method may be extended to other NIR-labeled imaging markers as a corollary to TLC used in assessment of radiopurity. The technique may provide a means for validating fluorescent imaging agents prior to human administration.

2. Materials and Methods

2.1 Reagents and equipment

Trastuzumab was provided in kind from Genentech (South San Francisco, CA). p-SCN-Bn-DTPA (2-(p-isothiocyanobenzyl)diethylenetriaminepentaacetic acid) was purchased from Macrocyclics, Inc. (Dallas, TX), and Sephadex G-25/PD-10 columns from GE Healthcare (Chalfont St. Giles, UK). IRDye 800CW was a gift from LI-COR Biosciences (Lincoln, Nebraska). Dulbecco's phosphate buffer solution, 1X, without calcium or magnesium, without phenol red, was purchased from MediaTech, Inc. (Manassas, VA). Tris-HEPES-SDS running buffer (NuSep #BG163) and 12% acrylamide gels (NuSep #NH11-012) were purchased from ISCBioExpress (Kaysville, UT). Methanol and 1M potassium phosphate buffer, pH 9.0, as well as NaHCO₃, sodium carbonate (#497-19-8), were purchased from Fisher Scientific (Houston, TX), and SDS sample buffer (#161-0737) from Bio-Rad (Hercules, CA). Zeba[™] desalting spin columns were purchased from Pierce (Rockford, IL). Equipment used included a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA), Labnet gel electrophoresis apparatus (#E2010-P, Edison, NJ), and an Odyssey imaging system (LI-COR, Lincoln, NE). Burdick & Jackson[™] high purity water (#365-4) and EMD sodium chloride (#EMD7710) were purchased from Cardinal Health (Houston, TX). Sodium phosphate dibasic heptahydrate (#S373-500) and sodium phosphate monobasic monohydrate (#S369-500) were both purchased from Fisher Scientific (Houston, TX). HPLC-grade phosphoric acid (#A260-500) was also purchased from Fisher Scientific. Sodium azide (#438456-5G) was purchased from Sigma-Aldrich (Atlanta, GA). A Hitachi LaChrom Elite preparative HPLC system with a L-2200 LaChrom Elite autosampler, a L-2455 LaChrom Elite diode array detector, and a Prep 24 solvent delivery system was used. A TSK-Gel® SW Series 6000XL μ m size exclusion column (#808541) and a TSK-Gel® SW GFC 7 μ m guard column (#808543) were purchased from Sigma-Aldrich (Atlanta, GA).

2.2 Reagent preparation

Trastuzumab was coupled with DTPA and IRDye 800CW, using the following procedure modified from the protocol described by Sampath *et al.*, 2007 [32]. Trastuzumab was dissolved in 50 mM NaHCO₃ (pH 8.0) at a concentration of 20 mg/mL and then mixed with 2X molar excess of the chelator, p-SCN-Bn-DTPA. After agitation of the mixture at room temperature for 30 min, the conjugated complex was loaded onto a Sephadex G-25/PD-10 column to remove unbound DTPA. The purified DTPA-trastuzumab was either stored at -20°C or immediately used for further IRDye 800CW conjugation. (DTPA)_n-trastuzumab was diluted to 1 mg/mL in PBS (pH 7.4), and 1M potassium phosphate buffer (pH 9.0) was added to the antibody to adjust the pH to 8.5. The molar IRDye 800CW-to-trastuzumab ratio ranged from 1 to 10. The conjugation reaction was performed at room temperature for 2 hours, protecting the vial from light. Separation of conjugate from free dye was performed by using a Pierce Zeba[™] desalting spin column or a PD-10 desalting column.

2.3 Reagent quantification

Protein concentration (mg/ml) and dye/protein ratios of conjugates were determined using UV/visible spectrophotometry with a Beckman DU800 spectrophotometer and calculations

as recommended by LI-COR (IRDye 800CW high molecular weight protein labeling kit, part # 928-38040).

2.4 HPLC Method

Five samples were prepared for size exclusion chromatography (SEC) HPLC analysis. Trastuzumab (100 µg injection), IRDye 800CW (10 µg injection), (DTPA)_n-trastuzumab (10 µg injection), and (DTPA)_n-trastuzumab—(IRDye 800)_m (10 µg injection) were all diluted with HPLC-grade water. DTPA (20 µg injection) was diluted with the mobile phase due to a major inflection peak seen when DTPA was dissolved in HPLC-grade water. The SEC mobile phase consisted of 0.0022M sodium phosphate buffer (pH 7.4) with 0.14M NaCl and 0.05% NaN₃. An isocratic run was performed through the size exclusion column for 20 minutes at 1 ml/min for each sample.

2.5 Gel sample preparation

A solution of prepared conjugate ((DTPA)_n-trastuzumab—(IRDye 800)_m) was diluted to 0.33 mg/ml. Fourteen µl of this solution was added to 56 µl of sterile, deionized, distilled water. Ten µl aliquots of this dilution were then placed into each of six 1.5-ml Eppendorf polyethylene tubes (the resulting amount loaded onto each gel lane was 2.21 pmoles). Unconjugated IRDye 800CW was prepared by diluting a 0.1 mg/ml solution of the dye (in sterile water) 1:250, 1:375, 1:500, 1:750, 1:1000, and 1:2500, and using water (no dye, for background subtraction) only. Ten-µl samples of these dilutions were added to the tubes containing conjugate. The resulting picomole amounts of free dye loaded per gel lane were 0.858, 0.572, 0.429, 0.286, 0.214, and 0.086. 20 µl of Laemmli/SDS sample buffer was added per tube. The tubes were closed and heated at 95°C for 5 minutes.

2.6 SDS-PAGE and imaging

Twenty µl of each of the spiked conjugate solutions were loaded into each of six spaced wells on 12% polyacrylamide gels, and electrophoresis was performed in Tris/HEPES/SDS running buffer for 45 minutes at 100 volts. Gels were removed from the plates and placed directly onto the screen of a LI-COR Odyssey infrared imaging system for measurement of fluorescent intensity at 800 nm at a machine setting of L2.0 intensity. Alternatively, the gels were imaged using illumination light from a laser diode (85 mA and 80 mW for 785-nm light, DL7140–201, Sanyo) that expanded to a circular area approximately 8 cm in diameter. The reemitted fluorescent light was collected with an EMCCD camera. Filter sets used included a band-pass filter with an 830-nm center wavelength and an optical density of greater than 3 at 785 nm (ANDV8483; Andover Corp., Salem, New Hampshire) for collecting IRDye 800CW fluorescence, and a holographic filter with an optical density of greater than 6 (HSP 785.0; Kaiser Optical Systems, Ann Arbor, Michigan) for rejecting backscattered and reflected excitation light. Image acquisition was accomplished with V++ software (Total Turnkey Solutions, Sydney, Australia). The integration time for fluorescence images was 800 ms.

2.7 Data analysis

The 16-bit tiff image file from the LI-COR Odyssey system was imported into Image J software (NIH). A region of interest (ROI) of defined pixel size was selected, and integrated intensity measurements were made of each gel band. The total fluorescent counts were acquired using the integrated density setting, since this parameter is not dependent upon a uniform ROI size for all bands analyzed. The gel band associated with (DTPA)_n-trastuzumab—(IRDye 800)_m corresponded to ~149 kDa, and free IRDye 800CW was found at ~1000 Da. The intensity of each free dye band was divided by the intensity of the corresponding conjugate band to obtain the molar percentage of free dye in the conjugate/dye preparation.

The ratios of free dye determined from these calibrations were then compared to the expected ratios of free dye to conjugate computed from spectrophotometric determination (Section 2.3 above).

3. Results

3.1 Assay Development

3.1.1 Identification of gel bands—Figure 1a shows a representative gel, with (DTPA)_n-trastuzumab—(IRDye 800)_m conjugate and free dye bands marked with arrows. The conjugate molecular weight is ~149 kDa, and the free dye molecular weight is 1.1 kDa. Addition of beta-mercaptoethanol to the loading buffer, however, produced another band at ~2 kDa (Figure 1b). The extra band at ~2 kDa may be a product of beta-mercaptoethanol forming an adduct with indole rings of the IRDye 800CW, possibly resulting in substitution at the phenoxy group of the dye, with subsequent linking of dye molecules to form dimers or other multimers (Dan Draney, LI-COR Biosciences, personal communication). All subsequent measurements were conducted without the use of beta-mercaptoethanol, so that true free dye bands were easily identifiable, and selectivity (the ability of an assay to measure the analyte of interest in the presence of other constituents in the sample), was properly addressed.

3.1.2 Practical considerations—Figure 2 shows the approximately linear increase in fluorescence intensity per mole of conjugate per dye/protein ratio of conjugates, indicating minimal to nonexistent quenching at higher dye/protein ratios. Figure 3a illustrates the image of a SDS page gel of IRDye 800CW in adjacent and spaced lanes on a 12% acrylamide gel. Standard deviations of integrated intensities were reduced when spaced lanes were used, as the diffusion of dye into adjoining lanes was avoided. Figure 3b shows three images of the same gel, obtained immediately, ten minutes, and 30 minutes after electrophoresis. At times greater than 10 minutes following electrophoresis, gel imaging resulted in leaking of the small IRDye 800CW molecule out of gel lanes. Gel running temperature (4°C versus 21°C) did not appreciably affect the standard deviations, nor did percentage of acrylamide in gels (10%, 12%, 4–20% gradient).

3.1.3 HPLC Fractionation Attempts—Figure 4 shows HPLC histograms of size exclusion chromatography of trastuzumab, DTPA, IRDye 800CW, (DTPA)_n-trastuzumab,

and (DTPA)_n-trastuzumab—(IRDye 800)_m. Retention times were 9.26, 12.39, 12.51, 8.85, and 8.80 minutes, respectively.

3.2 Assay validation

After developing and testing the specificity of the SDS-PAGE assay, we conducted a series of validation experiments to characterize the robustness of the assay to determine the linearity, limits of detection and quantification, precision, and accuracy.

3.2.1 Linearity of calibration standard curve—One parameter of method validation is linearity, which can be analyzed by producing a regression line for a plot of analyte concentration *versus* fluorescence (33, 34). The linearity of this method was evaluated by linearly regressing the fluorescence intensity *versus* moles of IRDye 800CW in five calibration standards. The average correlation coefficient of 0.9999 from six standard curves provided a “goodness of fit.” Figure 5 shows three standard curves, along with the regression equations. Precision for individual points on the curve, as determined by the coefficient of variation (% CV) values, ranged from 2.3 to 15.8%.

3.2.2 Lower limit of detection—Detection of the lower limit can be based on the standard deviation of the blank [33, page 11]. The lower limit of detection (LOD), used to define sensitivity, was measured by assaying 6 replicates of zero (H₂O added instead of dye) four times. The grand mean fluorescence intensity and pooled standard deviation of zero were calculated. The mean of each standard (in linearity assay, Figure 5) was used for the standard curve, and the mean signal of zero plus two standard deviations, read in dose from the standard curve, is the LLD; that is, the smallest dose that is not zero with 95% confidence. The lower limit of detection of free IRDye 800CW in this assay was found to be 7.1×10^{-3} pmoles. The calculations are shown in Figure 6. LOD (limit of detection) is the same as LLD (lower limit of detection) and the detection limit, and is the lowest quantity of a substance distinguishable from the absence of the substance, within a confidence limit, in this case 1%.

3.2.3 Lower limit of quantification for molar percentage of free dye determination—The lower limit of quantitation (LLOQ), according to the FDA’s “Guidance for Industry, Bioanalytical Method Validation” [34], is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision, and should serve as the lowest concentration on the standard curve, if the analyte response at the LLOQ is at least 5 times the response compared to blank response, and the analyte response is identifiable, discrete, and reproducible. The lower limit of quantification of free IRDye 800CW in this assay for free dye determination is 0.086 pmoles.

3.2.4 Upper limits of quantification and detection—According to the FDA’s “Guidance for Industry, Bioanalytical Method Validation [34],” the highest standard will define the upper limit of quantification (ULOQ) of an analytical method, and should be “the highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy.” Figure 7 shows that the highest standard before encountering pixel saturation and loss of accuracy when measuring fluorescence intensity is 17.15 pmoles. The

upper limit of quantification of free IRDye 800CW in this assay is 17.15 pmoles. The upper limit of free dye that could be accurately detected (ULOD) in 12% Tris-glycine gels, using a LI-COR Odyssey system at a reading intensity of L2.0, was also determined. At this setting, pixel saturation occurred above 18.5 pmoles. Individual pixel intensity measurements of dye concentrations over this limit would underrepresent high-intensity (>65,500) pixels, yielding inaccurate gel band intensities.

3.2.5 Precision and accuracy of determination of percentage of free dye in conjugate—We quantified free dye moles of standard amounts of free dye (representing 3–38% of the molar percentage of bound dye in (DTPA)_n-trastuzumab—(IRDye 800)_m conjugates) run together in gel wells with known amounts of conjugates, and determined their relative molar percentages. We used conjugate samples with two different dye/protein ratios to see if this ratio affected the assay. Figure 8a shows results of quantitation for a conjugate sample with a dye/protein ratio of 2.27, and Figure 8b shows results for a conjugate sample with a dye/protein ratio of 1.01. Accuracies of determination are shown in graphs depicting expected and actual percentages. Precisions of fluorescence intensities, defined as the % CVs, were less than 20%.

4. Discussion

Many experimental agents can be tested for purity using HPLC analysis. Unfortunately, we found that distinct quantification of free dye using a size exclusion HPLC method was not possible, because free DTPA and free IRDye 800CW eluted at almost identical timepoints. We then tested a polyacrylamide gel separation-based assay to quantitate unbound dye in our conjugate preparations.

In the process of developing and validating this assay for free dye determination in (DTPA)_n-trastuzumab—(IRDye 800)_m conjugates samples, we first determined whether several assay features affected validation parameters. Gel sample preparation reagent, for example, affected the specificity of the assay. When beta-mercaptoethanol was included in sample running buffer, an extra band appeared near the “true” free dye band. Accuracy (nearness to expected values) and precision (%CV) were affected by gel band spacing, since standard deviations greatly increased when samples were run in adjoining lanes, allowing overlap of fluorescence. Quenching did not seem to occur in conjugates with dye-to-protein ratios over 2.0, so this method could be applied to such conjugates. The lower limit of detection was calculated here using a zero/standard deviation method, and agreed with our efforts to identify the lowest identifiable, non-zero amount of free dye (data not shown). The lower and upper limits of quantitation, as well as the upper limit of detection, were set at values according to FDA guidance document recommendations [34].

After developing a standard operating procedure for this assay, we then validated the assay by testing linearity and comparing actual to expected values of fluorescence intensity. %CVs of fluorescent intensity values for dye amounts within the limits of quantitation were under 20%, and the linearity of fluorescent intensity versus dye amounts was excellent ($R^2=0.9999$).

In this study, we focused on using IRDye 800CW for two reasons. First, the dye has an emission wavelength in the optimal near infrared range for imaging mammalian tissue, which is ~800 nm, where absorbance by hemoglobin, melanin, and water are minimal. Second, we have performed pre-clinical safety and toxicity studies on IRDye 800CW, in anticipation of extending studies into humans. This assay could be adapted for use with other optical dyes, if a suitable instrument or method is available for fluorescence intensity measurement of gel bands, which could be excised and measured in wells of plates on fluorescence plate readers.

The use of near infrared optical agents for noninvasive *in vivo* imaging holds great promise. Figure 9 shows images obtained using trastuzumab with both optical (IRDye 800) and nuclear (^{64}Cu) labels in a murine model of breast cancer. Dual-labeled imaging agents can help ascertain the accuracy, usefulness, and appropriate applications for optical imaging moieties, as compared to and in addition to nuclear agents. To our knowledge, this is the first purity validation study of an optical imaging agent conjugate. As optical imaging expands into clinical applications, such validations will be necessary to ensure the purity of agents used.

5. Conclusions

A crucial parameter for *in vivo* molecular imaging is the minimization of the amount of free, unconjugated dye or tracer in the conjugate preparation to ensure detection of only conjugate-binding cells, and not free dye or free dye-binding cells. In this study, we describe a simple and rapid method for quantifying free/unconjugated dye in $(\text{DTPA})_n$ -trastuzumab— $(\text{IRDye } 800)_m$ conjugate samples, and address validation parameters of accuracy, precision, selectivity, range, and limits of detection and quantitation.

We found the assay to be accurate and valid for free IRDye 800CW amounts between 0.086 and 0.86 pmoles of free dye that may be present in a 2.2 pmole conjugate sample. Alternate gel lanes must be used to prevent spillover fluorescence from neighboring gel lanes, beta-mercaptoethanol must be omitted from the SDS sample buffer, and gels must be imaged within ten minutes of electrophoresis to prevent diffusion of the free dye molecule. The mean lower limit of quantification for this assay for linearity was 0.086 pmoles, and the upper limit was 17.15 pmoles. The lower limit of detection of dye was 7.1×10^{-3} pmoles, and the upper limit of detection of dye in this assay was 18.5 pmoles. This method may be applicable to other near infrared dye-conjugated imaging agents in which HPLC assessment of purity is not feasible.

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Figure 1.

(a) Image of 12% gel with bands corresponding to varying picomoles of free dye alone (left) and when added to 16.5 picomoles of conjugate (right). (b) The presence of beta-mercaptoethanol in loading buffer results in an extra band on the separation gel.

Figure 2.

Fluorescence intensity per mole of conjugate *versus* dye-to-protein ratio for varying ratios. Each point corresponds to the mean of 6 samples, and the error bars denote standard deviations. Conjugates with dye/protein ratios higher than 1 exhibit approximately linear fluorescence intensity per mole of conjugate ($R=0.8739$). Graph shown is representative of three separate measurements of conjugates of varying dye-to-protein ratio.

Figure 3.

(a) Alternate (spaced) gel lane use prevents spillover fluorescence from neighboring gel lanes. Each gel lane contains 4.29×10^{-1} pmoles IRDye 800CW. For spaced lanes, the average fluorescence intensity was 2.55×10^6 arbitrary units, with a standard deviation of 13.26×10^3 units, while for the adjacent lanes, the average fluorescence intensity was 2.50×10^6 units, with a standard deviation of 95.98×10^3 units. (b) Free dye leakage occurs if time lapse between end of gel run and gel imaging is excessive.

Figure 4.

(a) HPLC chromatogram of trastuzumab at 280 nm with a retention time of 9.26 minutes. (b) chromatogram of DTPA at 280 nm with a retention time of 12.39 minutes. (c) chromatogram of IRDye 800CW at 780 nm with a retention time of 12.51. (d) chromatogram of trastuzumab conjugated with DTPA at 280 nm with a retention time of 8.85 minutes. (e) chromatograms of trastuzumab conjugated with DTPA and IRDye 800CW at 280 nm (1) and at 780 nm (2) with a retention time of 8.80 minutes.

Figure 5.

Fluorescence intensity *versus* pmoles of free dye. Symbols denote average of 6 samples and measurements, and error bars correspond to standard deviations. The calibration curve was linear ($R=0.9999$) for 0.086 to 17.15 pmoles dye, when fluorescence was read at an L2.0 intensity setting of Odyssey. Graphs show three separate experiments which yielded similar results.

Figure 6.

Calculations for lower limit of detection. The lower limit of detection (LOD), used to define sensitivity, was measured by assaying 6 replicates of zero (H_2O added instead of dye) four times. The grand mean fluorescence intensity and pooled standard deviation of zero were calculated. The mean of each standard (in linearity assay, Figure 5) was used for the standard curve, and the mean signal of zero plus two standard deviations, read in dose from the standard curve, is the LLD. The lower limit of detection of free IRDye 800CW in this assay was found to be 7.1×10^{-3} pmoles.

Upper limit of detection determination:

	trial 1		trial 2		trial 3	
pmoles dye/well	fluorescence intensity/well	max. intensity per pixel	fluorescence intensity/well	max. intensity per pixel	fluorescence intensity/well	max. intensity per pixel
0.4	536198	1337	579542	1331	422686	1556
4.3	5663993	13876	6030780	13197	5434371	21282
8.6	11537459	27941	12314069	26416	11679995	34279
10.7	14702107	36137	15988280	35127	15737388	37575
21.5	29218271	65535	31994227	65535	25564085	65535
42.9	44854855	65535	46180643	65535	31736670	65535

Figure 7.

Determination of upper limit of detection (ULOD). Figure 7 shows that the highest dye amount before encountering pixel saturation (65,535 fluorescence intensity maximum, arbitrary units) and loss of accuracy when measuring fluorescence intensity is 18.5 pmoles.

Figure 8.

(a) Actual mean and expected mean free dye percentages (ratios) of combined dye and conjugate samples for a conjugate sample with dye-to-protein ratio=2.27. (b) Actual mean and expected mean free dye percentages (ratios) of combined dye and conjugate samples for a conjugate sample with dye-to-protein ratio=1.01. Precisions are represented by % CVs. n=6. Each graph shows three separate experiments that yielded similar results.