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Articles

Simple, Rapid Chemical Labeling and Screening of Antibodies with Luminescent Peptides

Virginia A. Kincaid,* Hui Wang, Casey A. Sondgeroth, Emily A. Torio, Valerie T. Ressler, Connor Fitzgerald, Mary P. Hall, Robin Hurst, Monika G. Wood, Julia K. Gilden, Thomas A. Kirkland, Dan Lazar, Hsu Chia-Chang, Lance P. Encell,* Thomas Machleidt, Wenhui Zhou, and Melanie L. Dart*

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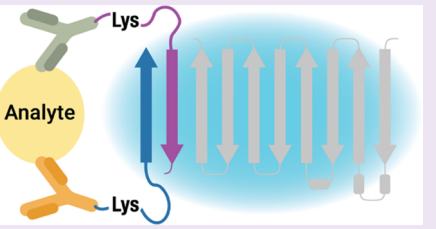
ABSTRACT: Sensitive and selective detection assays are essential for the accurate measurement of analytes in both clinical and research laboratories. Immunoassays that rely on nonoverlapping antibodies directed against the same target analyte (e.g., sandwich enzyme-linked immunosorbent assays (ELISAs)) are commonly used molecular detection technologies. Use of split enzyme reporters has simplified the workflow for these traditionally complex assays. However, identifying functional antibody pairs for a given target analyte can be cumbersome, as it generally involves generating and screening panels of antibodies conjugated to reporters. Accordingly, we sought a faster and easier reporter conjugation strategy to streamline antibody screening. We describe here the development of such a method that is based on an optimized ternary NanoLuc luciferase. This bioluminescence complementation system is comprised of a reagent-based thermally stable polypeptide (LgTrip) and two small peptide tags (β9 and β10) with lysine-reactive handles for direct conjugation onto antibodies. These reagents enable fast, single-step, wash-free antibody labeling and sensitive functional screening. Simplicity, speed, and utility of the one-pot labeling technology are demonstrated in screening antibody pairs for the analyte interleukin-4. The screen resulted in the rapid development of a sensitive homogeneous immunoassay for this clinically relevant cytokine.



INTRODUCTION

Reporter-labeled antibodies are invaluable tools for measuring analytes, both in clinical and research environments.[1−3] As affinity reagents, they can be used in a variety of assay systems and formats. Homogeneous immunoassay formats present inherently simple workflows, making them ideal for high-throughput screening applications. These types of assays require affinity reagents that can bind to distinct epitopes on the same antigen and generate a specific, measurable reporter signal without the need for wash steps. Further, these methods must be compatible with a variety of biological sample types. While antibodies are readily available for many analytes of interest, simple, modular reporter conjugation systems that enable rapid labeling and functional screening are needed to accelerate the current state of homogeneous molecular detection immunoassay development.[4]

Bioluminescence-based affinity reagents are of interest for molecular detection assays because they provide low background, high sensitivity, and broad dynamic range. NanoLuc luciferase (Nluc) has emerged as a bioluminescence enzyme of choice because it is small and produces a bright, sustained signal.[5,6] Nluc is also extremely stable, making it particularly well suited for use in biochemical reagents. Split versions of Nluc have been applied successfully as reporter moieties in numerous homogeneous molecular detection immunoassays.[4,7−14] Genetic fusions between antibodies and reporters have been effective;[7,13] however, knowledge of protein sequence for commercial antibodies is required to build such constructs. Two approaches have recently been used for chemical conjugation between Nluc reporter sequences and antibodies. In one case, complementary binary fragments of Nluc (i.e., NanoBiT[15]) were tethered to chloroalkane-labeled antibodies[16] using genetic fusions to HaloTag.[10,17] Following a similar concept, but also providing the means for ratiometric detection, the same binary Nluc reporters were fused to a modified protein G to enable non-natural amino acid-based, immunoglobulin G (IgG) Fc region-specific photoconjugation onto full-length, subclass IgG antibodies.[4] While these and other cited methods provide efficient labeling of antibodies, the size of the HaloTag- or protein G-based fusion labels (as large as 40−50 kDa) could compromise antibody function. Of greater concern, these approaches may require time-consuming, higher complexity labeling and cleanup protocols that are not compatible with high-throughput screening.

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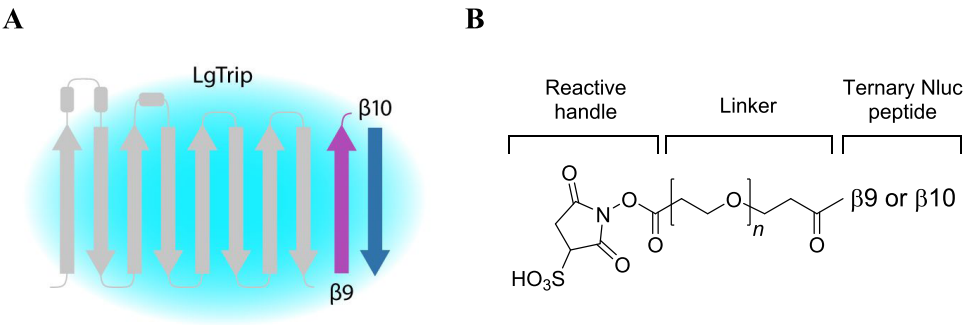
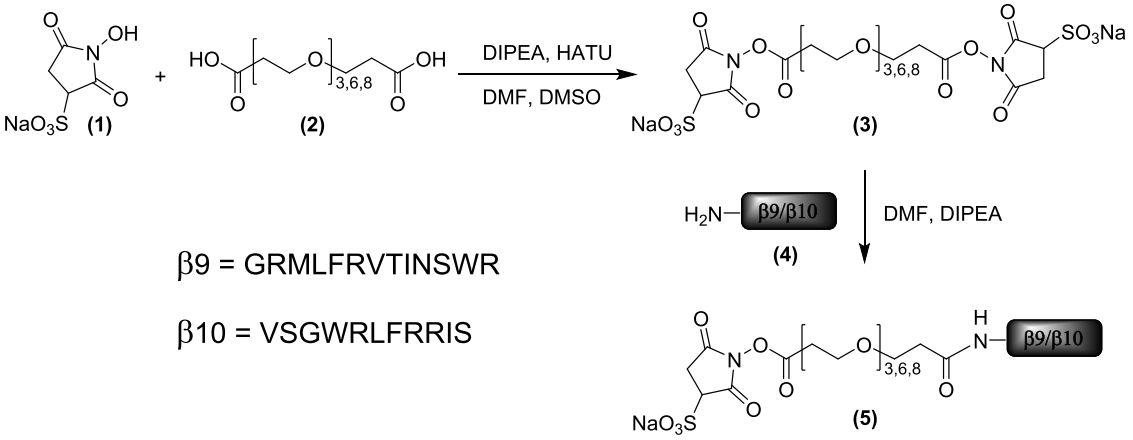


Figure 1. Peptide label design. (A) The ternary Nluc system possesses two small peptides that correspond to strands 9 (β9, purple) and 10 (β10, blue) of the 10-strand luciferase β-barrel. The remaining strands (1−8) represent a complementary 17 kDa polypeptide (LgTrip). (B) Chemical labels derived from ternary Nluc peptides contain lysine-reactive *N*-hydroxysulfosuccinimidyl ester handles appended to the N-terminus of β9 or β10 peptide via a poly(ethylene glycol) (PEG) linker of varying length.

Scheme 1. Synthetic Route for Chemical Modification of Ternary Nluc Peptides with sulfo-NHS[a]



[a](1) sulfo-NHS; (2) bis-PEG-acid; (3) bis-PEG-sulfo-NHS; (4) NH_2 peptide; and (5) labeling product.

We recently described a sensitive, ternary Nluc reporter amenable to homogeneous immunoassays for point-of-need diagnostic testing in a variety of sample matrices.[9] The three-component bioluminescence complementation system consists of a reagent-based polypeptide (LgTrip 3546) comprising Nluc strands 1−8 and two small peptide tags, 521 and HiBiT (forming the C-terminal strands β9 and β10, respectively, in a full length, reconstituted enzyme) (Figure 1A). In the previous report,[9] genetic fusions between each peptide and HaloTag were constructed, overexpressed, purified, and used to label compatible antibody pairs against the same antigen. The small reporter peptides are brought into proximity when antibodies recognize and bind to a target antigen, and in the presence of LgTrip and luminogenic substrate, a bioluminescence signal is generated. An important property of ternary Nluc is that none of the three components have inherent luminescence activity. As a result, LgTrip can be present at high, optimal levels (independent of reporter peptide concentrations) in the reagent to efficiently drive the formation of the reconstituted luciferase enzyme.

As HaloTag-based ternary Nluc labeling still requires multistep, multiday protocols (Figure S1), we aimed to create a chemical conjugation strategy for directly appending ternary Nluc peptide reporters onto antibodies in a simple and fast workflow. Such a technology would enable rapid generation and screening of large panels of labeled antibodies against analytes of interest. Herein, we report the design, synthesis, and optimization of ternary Nluc sequences and lysine-reactive reporter peptide derivatives to meet these criteria. The resulting technology enables simple, rapid, high-throughput antibody labeling and screening using small, sensitive peptide reporters. Utility and performance of the new system matched or exceeded that of our HaloTag-based method,[9] doing so with greatly collapsed antibody labeling and screening protocols. The integrated, streamlined workflow facilitated rapid screening of interleukin-4 (IL-4)-specific antibody pairs, culminating in a robust detection assay for this model target analyte.

RESULTS AND DISCUSSION

Design of Ternary Nluc Peptides for Labeling Antibodies. The β9 and β10 peptides (521 and HiBiT, respectively) from ternary Nluc (Figures 1A and S2A and Scheme 1) are desirable as reporter tags due to their small size (11−13 amino acids).[9] Generally, small tags are less likely to impede target function sterically or chemically after conjugation. Additionally, the small size of peptides makes them conformationally independent (i.e., lacking higher-order structure). This feature allows peptides to be utilized as chemical matter in the harsh conditions required for synthetic modification. Unlike labeling methods that require genetic manipulation, chemical labeling approaches omit the need for upstream cloning, protein expression, and purification, making them more modular and flexible. Though the sequence and physical properties of peptides will ultimately play a role, we

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envisioned a general strategy where a chemical handle could be synthetically attached to the β9 and β10 peptides to allow direct conjugation onto protein targets in a single-step reaction.

In selecting a handle for conjugation of ternary Nluc peptides onto antibodies, we focused our efforts on the well-established *N*-hydroxysuccinimidyl ester (NHS) chemical family that has been used extensively for protein labeling (Figure 1B).[18,19] NHS reacts with solvent-exposed primary amines, such as ε-amino lysine side chains, to form stable amide bonds with target proteins. To eliminate the occurrence of intra- and intermolecular reactions between peptides and NHS groups, it was imperative to remove lysine residues from the β9 521 (GKMLFRVTINSWK) and β10 HiBiT (VSGWRLFKKIS) peptide sequences. We substituted arginine for each lysine residue in both peptides and observed minimal impact on the complementation binding parameters and luminescence signal of the ternary Nluc reporter (Figure S2B,C). These results indicated that the arginine-based ternary Nluc peptides (referred to as β9 and β10 in the remainder of this work) were good candidates for chemical modification with NHS and subsequent conjugation to antibodies.

Synthetically attaching NHS groups to β9 and β10 presented several challenges. Initially, we took a two-step synthetic approach, first appending a bis-carboxylic acid linker to the free peptide N-terminus and then attempting to convert the second carboxylic acid to NHS under standard coupling conditions (Scheme S1A).[20,21] This approach failed to give the desired products, suggesting that the activated carboxylic acid intermediate may have reacted with additional amino acid side chains on the peptide (e.g., arginine, serine). We therefore used an alternate synthetic strategy where both carboxylic acid groups on the linker were converted to NHS and then attached to the peptide under basic coupling conditions (Scheme S1B). Minimal product was isolated using standard NHS reactive groups, likely due to instability. However, a sulfonated derivative of NHS proved more stable under basic conditions, resulting in the desired ester coupling products (30−40% yield; Scheme 1). Final label products were lyophilized, resuspended in anhydrous dimethylformamide (DMF), and stored at −20 °C. No evidence of label degradation was observed under these storage conditions. The presence of H_2O is detrimental to the integrity of the NHS groups over time, and so it is important to use an anhydrous solvent to achieve maximum stability.

The synthetic strategy enabled the modular introduction of poly(ethylene glycol) (PEG) linkers (Figure 1B), ultimately enabling us to examine the impact of linker length (i.e., PEG3, PEG6, or PEG8) on reporter utility and performance (i.e., presentation of peptides on the surface of antibodies and luciferase reconstitution) in functional assays (see the Supporting Information; specifically Figures S3 and S4). These experiments showed that either the PEG6 or PEG8 linker is a viable option, thus validating the choice of the PEG6 linker for the experiments described here.

Protocol for Conjugating Ternary Nluc Peptides onto Antibodies. To develop an antibody labeling protocol, we employed the previously characterized anti(α)-interleukin-6 (IL-6) model system.[9] Based on previous reports of successful labeling of lysines found in non-antigen-binding regions of antibodies,[22−24] we chose to use a molar ratio of 10:1 (label:antibody) for our conjugation reactions with the expectation that this would yield antibodies containing 1−10 labels. This seemed a reasonable target based on recent accounts of highly sensitive split Nluc-based immunoassays using genetic fusions, where only a single reporter peptide is necessary for each molecule of affinity reagent.[7−9,11,25,26]

Due to the small size of our labels (<3 kDa), it was difficult to resolve individual label additions to the significantly larger antibodies (150 kDa) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, it became easier to visualize labeling upon reducing disulfide bonds in the antibodies to yield smaller proteins (heavy chain = 50 kDa; light chain = 25 kDa; Figure S5). Serial label additions to the light chain were observable as distinct bands. In contrast, the labeled heavy chain appeared as a smear. As a result, determining the degree of labeling by SDS-PAGE proved more qualitative than quantitative. Nonetheless, as expected, conjugation reactions produced a heterogeneous mix of products with varying degrees of labeling. Labeling was generally inefficient, as there remained significant amounts of unlabeled material in the reactions. Increasing labeling efficiency is a clear objective for continuing efforts to improve the sensitivity and performance of the overall methodology. Both αIL-6 antibodies displayed labeling on their heavy and light chains, though the 505E antibody clone showed a preference for heavy chain labeling. The distinct labeling patterns observed between the two antibodies were likely due to variations in the location and local pH values of exposed lysine residues.

Typically, alkaline buffers are recommended to activate lysines on target proteins for NHS conjugation.[18] However, most commercially sourced antibodies are provided in phosphate-buffered saline (PBS) at neutral pH. Labeling of a commercial antibody in its provided storage solution would be preferred over having to carry out a buffer exchange for ease of workflow. Thus, we compared the quality of antibody conjugation products generated in alkaline sodium bicarbonate buffer (pH 8.5) or the provided PBS storage buffer at neutral pH. Perhaps unsurprisingly, conjugation reactions conducted in alkaline buffer yielded products with a higher degree of labeling (i.e., labels per molecule) and with greater overall labeling efficiency based on decreasing amounts of unconjugated antibodies (Figure S5).

Optimization of Ternary Nluc LgTrip. Prior to any functional testing of the methods developed thus far for labeling antibodies with ternary Nluc peptides, we turned our focus to optimizing the LgTrip component of the complementation system. As a critical part of the ternary Nluc detection reagent, an ideal LgTrip polypeptide needs to be stable on the bench or in the field, amenable to different storage conditions, and functional across a variety of environmental conditions and sample types. Though already relatively stable, we attempted to maximize the physical stability of LgTrip 3546 by constructing and screening a library containing all of the possible single amino acid changes across the full protein sequence. The recently reported LgTrip 3546[9] was identified by screening mutant libraries created by error-prone polymerase chain reaction (PCR), a method known to have mutational bias.[27] We reasoned that a more comprehensive and diverse single amino acid substitution library would allow better examination of sequence space and enable the identification of LgTrip variants with even greater stability. In turn, we built and screened the more comprehensive library for luminescence output and thermal stability as previously described.[9] Mutants displaying greater than 40% improvement

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in luminescence were sequenced, genetically combined, and rescreened.

Our screening efforts led to the discovery of a triple mutant, LgTrip 5146 (LgTrip 3546 + R112H + K123E + T144D) (Figure S6), which produced more than 3-fold higher luminescence signal intensity in bacterial lysates (Figure S7A). However, purified protein LgTrip 5146 did not possess higher specific activity (i.e., maximum luminescence signal intensity per mole of enzyme) than LgTrip 3546 (Figure S7B–G), suggesting that the increased signal observed in lysates was due to improved expression (i.e., accumulation and/or persistence of LgTrip in *Escherichia coli*). Elevated levels of protein inside cells could result from more efficient folding, increased solubility, or increased biophysical stability. Indeed, LgTrip 5146 displayed improved thermal stability (melting profile shifted by 5 °C) relative to LgTrip 3546 (Figure S8A). As a result, LgTrip 5146 was twice as active as 3546 following exposures to 80 °C. After exposure to 90 °C, LgTrip 5146 maintained significant (25%) activity, while 3546 was essentially inactivated. The maintaining of activity following exposure to elevated temperature suggests that LgTrip 5146 possesses higher biophysical stability. This will likely translate to a more shelf-stable, robust reagent, a property likely to be of particular importance for use in high-throughput screening applications and point-of-need settings where refrigeration is not available.

In addition to producing bright luminescence, Nluc and many of its derivatives offer a stable, glow-type signal making it highly amenable to higher-throughput end point assay formats.[5] To examine whether the evolved ternary Nluc system maintained the glow kinetics associated with Nluc, we measured signal duration for the fully complemented ternary system. The results indicate that the ternary Nluc signal decayed slightly (1.5-fold) faster than that of Nluc (Figure S8B), but the half-life was still well over an hour (i.e., glow-type kinetics).

To try and understand the mechanism for its improved stability, we created a structural model of LgTrip 5146 bound to the β9 and β10 peptides (Figure S9). The model predicts that two of the three amino acid substitutions (T144D and K123E) are near the termini of the protein, but there is no obvious explanation for how these could be contributing to enhanced stability. It is noteworthy that neither LgTrip terminus is a native sequence, as the N-terminus contains a polyhistidine, and the C-terminus is a product of β-strands 9 and 10 removal. T144D and K123E could play a role in stabilizing the non-native termini. The third substitution, R112H, is on the β-barrel side opposite the termini, closer to the interface between LgTrip and the β9 and β10 peptides. However, neither the original arginine nor the substituted histidine appears capable of interacting with the complementary peptides. Regardless, as our current state-of-the-art version, LgTrip 5146 was utilized in all assays described in the remainder of this work because of its enhanced stability.

Determination of Critical Components of Antibody Labeling Protocol. To investigate the practical and functional significance of more efficient antibody labeling taking place in alkaline buffers (Figure S5), we performed the IL-6 analyte detection assay using antibody–ternary Nluc peptide conjugates prepared in either alkaline or neutral pH buffers. Washed conjugation products were titrated with saturating LgTrip 5146 (1 μM) in the presence and absence of IL-6 analyte. Upon assaying with luminogenic substrate, the antibody conjugate concentration that gave the largest assay dynamic range (signal over background, S/B) was 62.5 ng/mL for conjugates prepared in both conjugation buffers (Figure S10A). Despite the lower labeling efficiency observed by gel images, antibodies labeled in PBS (pH 7) were only 2.5-fold dimmer than conjugates prepared in sodium bicarbonate (pH 8.5) (Figure S10B). However, conjugates prepared in PBS also demonstrated lower assay background, resulting in a similar optimal assay dynamic range for both PBS and alkaline labeling conditions (Figure S10A).

Although PBS yielded less efficient labeling and thus conjugates with slightly less robust luminescent signal output, utilizing PBS as a conjugation buffer allowed omission of a buffer exchange step before antibody conjugation (i.e., because the antibodies could be labeled in their PBS storage buffer). Highly simplified labeling protocols are particularly desirable for collapsed screening workflows. Consequently, we chose to use PBS as the conjugation buffer for the remainder of our efforts with the aim of optimizing our protocol for antibody screening applications. Note that this decision was made under the assumption that once a compatible pair of antibodies is identified for a given target analyte, buffer exchange into alkaline conditions can and should be explored for conjugation and assay optimization.

To better understand the importance of conjugation reaction time, we used the same IL-6 detection system (505E and 5IL6 αIL6 antibodies paired with β9 and β10 labels, respectively) as a functional end point assay for monitoring ternary Nluc peptide conjugation progression over time. Single-pot labeling reaction samples were quenched at incremental time points by adding Tris (pH 7.5) to a final concentration of 100 mM (Tris reacts with any remaining free NHS ester groups). Crude conjugation products (i.e., unwashed) were assayed in the presence and absence of IL-6. IL-6-dependent signal generation was rapid, reaching maximum signal in less than 30 min of labeling (Figure 2A). This suggested that antibodies were labeled to functional saturation levels very quickly. More than a 100-fold dynamic range was also achieved within 30 min (Figure 2B). These results revealed two important features of the system: (1) antibody labeling is rapid, and (2) the resulting assay provides a robust, analyte-specific signal and broad dynamic range in a homogeneous, no-wash format. Note that the background signal (i.e., signal in the absence of IL-6) was reduced at longer time points. This could have been a result of free label molecules degrading, aggregating, or adsorbing to plastic over time.

To further examine the necessity for a post-conjugation wash step, the impact of free labels on background and assay performance was investigated by comparing αIL-6 antibody conjugates (generated from 1 h conjugation reactions in PBS) that either included or omitted a desalting wash step after label conjugation. Desalting is an established, efficient washing method for the removal of small molecules and peptides from solution. Due to the small size of our peptide labels (<3 kDa), we anticipated that desalting (i.e., microcolumn centrifugation) would provide a simple way to remove unconjugated labels from crude reaction mixtures. Using the IL-6 assay to examine crude versus desalted reactions, we observed that the washed antibody conjugates produced comparable IL-6-induced signal strength but with a 10-fold lower assay background (Figure 3). Thus, the removal of free label molecules was likely the primary source of reduced background and improved assay

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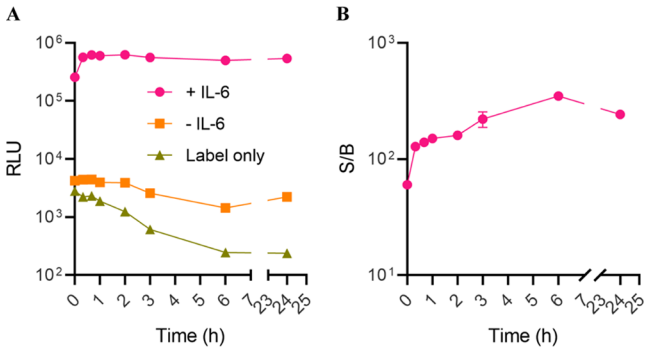

Figure 2. Labeling kinetics. A 10-fold molar excess of the label (67 μM) was added to 1 mg/mL (6.7 μM) αIL-6 antibodies in PBS. Antibodies 5IL6 and 505E were conjugated to β9 and β10, respectively. Minus antibody controls (i.e., label only) were run in parallel. Excess Tris (final [Tris] = 100 mM) was added to quench reactions. Time = 0 values were taken immediately after label addition. (A) Crude, unwashed β9 and β10 conjugate reaction mixtures from the indicated time points were assayed in cell culture media at 62.5 ng/mL (4.2 nM) antibody. Conjugate pairs were assayed in the presence (+IL-6) and absence (−IL-6) of 10 ng/mL IL-6 analyte. Mean relative light units (RLU) values (n = 2) of a representative experiment are shown with variability expressed as standard deviation (SD). (B) Assay dynamic range (S/B) was calculated for each time point by dividing the (+) IL-6 signal by the (−) IL-6 signal from panel (A).

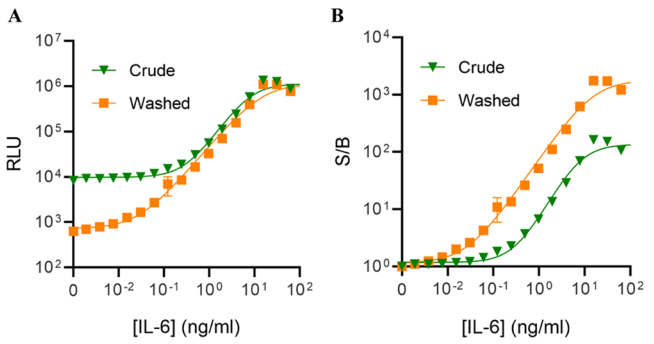

Figure 3. Conjugate cleanup improves assay performance but is optional for streamlining antibody labeling-to-assay workflow. Conjugation reactions were run for 1 h in PBS and quenched (final [Tris] = 100 mM). Conjugates were either set aside as crude reaction products or washed via desalting columns. β9 and β10 antibody conjugates were assayed at 62.5 ng/mL against a titration of IL-6 analyte in cell culture media. (A) Raw RLU and (B) signal over background (S/B) are plotted for the same data set. Mean values ± SD are shown (n = 3).

performance; however, there was evidence of higher molecular weight conjugates also being removed during the wash step (Figure S5). As a result, we cannot say with certainty that these species were not at least contributing to the background observed from the crude labeling reactions. If so, this suggests that antibody overlabeling could adversely affect functional performance. Overlabeled antibodies could present the ternary Nluc components with too many options for complex formation, potentially diluting out or slowing down formation of the most productive reconstituted luciferases. It is also entirely possible that increasing the number of labels per antibody will eventually compromise antibody functionality. Further, excess labeling could eventually lead to increased background complementation in the absence of an analyte.

The assay using washed conjugates provided a significantly improved dynamic signal range (3 orders of magnitude versus 2), increased sensitivity (limit of detection (LOD) of 1 pg/mL versus 78 pg/mL), and a linear range of detection that spanned more than 5 orders of magnitude (ULOQ = 123 ng/ml). These results indicate that this new methodology gives comparable or better performance relative to our previously reported assay using the HaloTag-based conjugation system with the same pair of antibodies.[9]

Perspective on Direct Ternary Nluc Peptide Labeling of αIL-6 Antibodies. Our intention was to develop a technology and methodology for direct labeling of antibodies with ternary Nluc peptide reporters to support antibody screening. The outcome of this work is a fast (3 h), simple (no antibody buffer exchange, no post-labeling wash steps) workflow that enables rapid labeling of antibodies with luminescent peptides and immediate, relatively high throughput screening for analyte detection activity (Figures 4 and

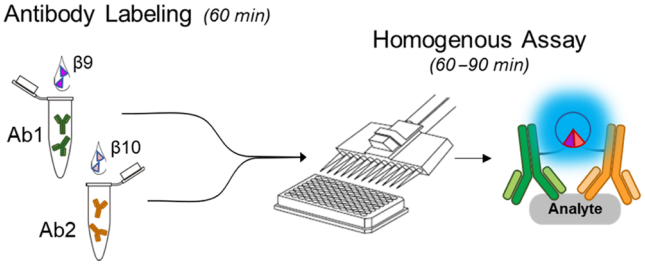

Figure 4. General workflow for conjugation and assay of antibody pairs using ternary Nluc peptide labels.

S11). Note that the simple and rapid format we have achieved is intended for screening purposes. For actual assay development, the same general approach can be applied but additional steps (i.e., buffer exchange for commercial antibodies, post-labeling wash) should be examined and likely implemented to optimize a given analyte detection assay.

Screening for Optimal αIL-4 Antibody Pairs. To assess whether we achieved our objective to create an antibody labeling methodology amenable to high-throughput screening of antibody pairs, we used the approach to try to identify an optimal pair of antibody conjugates for immunoassay-based detection of human IL-4. IL-4 is an important cytokine involved in immune system regulation.[28] A panel consisting of six commercially available IL-4-specific antibodies (Table S1) was conjugated to β9 and β10 peptide labels in PBS for 1 h. Unwashed, crude conjugation products were immediately moved into functional screening, assaying all possible pairings of β9 and β10 antibody conjugates at 250 ng/mL in the presence and absence of an IL-4 analyte. Note that we screened with a single concentration of IL-4 (50 ng/mL). However, the protocol is amenable to screening with multiple antibody conjugate and analyte concentrations by simply preparing additional reagents and using replicate plates. Note that testing additional analyte concentrations may be necessary when the properties of sourced antibodies (e.g., affinity, quality) are not well understood. In less than 3 h (30 min of hands-on time), we labeled and screened 36 combinations of β9- and β10-labeled antibodies. A variety of factors, including conjugation efficiency, antibody−antigen affinity, epitope proximity, and label positioning, will potentially contribute to assay performance. Taking all of these properties into account

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simultaneously with our screen, we identified the antibody pair, Ab5 and Ab6 (Invitrogen antibody clones E10022 and E10023, respectively), as providing the highest signal (Figure 5A) and dynamic range (Figure 5B). With this pair of antibodies, there was no preference for one orientation of the peptide labels over the other.

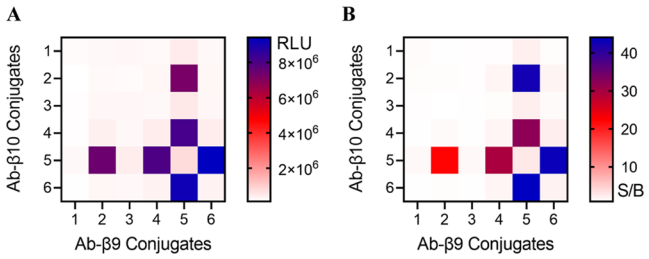

Figure 5. Rapid screen of IL-4-specific antibodies. A panel of six αIL-4 antibodies was conjugated to β9 and β10 labels and assayed in cell culture media ±50 ng/mL IL-4 analyte. Results of the screen are shown according to (A) light output (RLU) in the presence of the analyte and (B) assay dynamic range (S/B).

To ensure that unconjugated labels did not interfere with screening results, the entire panel of antibody conjugates was washed and rescreened for validation. After cleanup, Ab5 and Ab6 conjugate pairs remained the brightest with a large dynamic range (Figure S12A,B), corroborating our "no wash" assay development approach. Additionally, Ab1-4 conjugates were visualized by SDS-PAGE, confirming that their poor performance was not due to lack of labeling (Figure S12C,D).

We used the developed IL-4 immunoassay as a model system to examine the robustness of the ternary Nluc direct antibody labeling method. First, washed Ab5-β10 and Ab6-β9 hits from the αIL-4 screen were assessed by assaying against a titration of the IL-4 analyte. The washed conjugates displayed more than 20-fold improvement in LOD relative to crude conjugates (washed conjugates LOD = 3.5 pg/mL) (Figure 6).

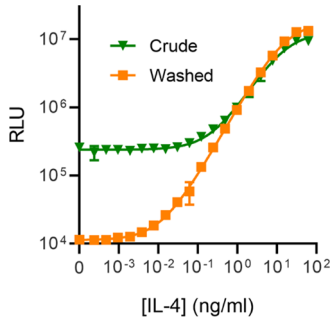

Figure 6. Characterization of IL-4 antibody screen hits Ab6-β9 and Ab5-β10. Washed and crude antibody conjugates were assayed at 250 ng/mL against a titration of the IL-4 analyte in cell culture media. Mean RLU values (n = 3) are shown with variability expressed as SD.

Next, three independent batches of Ab5-β10 and Ab6-β9 conjugates were generated. Each displayed similar conjugation products by SDS-PAGE as well as functional performance (Figure S13). These results indicate that antibody labeling with our technology/methodology is robust and reproducible.

Comparison to IL-4 Enzyme-Linked Immunosorbent Assay (ELISA). ELISAs are broadly used in clinical and research settings and are often considered to be the gold standard for molecular detection assays. However, ELISAs are heterogeneous, multistep assays that can be cumbersome and time-consuming (>6 h). In contrast, our IL-4 immunoassay is a homogeneous assay (i.e., uses a single reagent) and can be completed in less than 2 h. To assess the performance of our new approach compared to ELISA, we generated three independent IL-4 standard curves using either our screening-derived IL-4 immunoassay or a commercially available IL-4 ELISA (Quantikine, R&D Systems) (Figure S14A–D). The data revealed the ternary Nluc assay to be far less noisy at low concentrations of analyte compared to the ELISA, and as a result, it was nearly 10-fold more sensitive. We also demonstrated that the ternary Nluc system could detect low levels of secreted IL-4 from stimulated Th2 immune cells (Figure S14E). These benchmarking experiments indicate that direct antibody labeling with ternary Nluc reporter peptides can be utilized to quickly generate homogeneous immunoassays for target analytes and with optimization ultimately provide assays that are competitive with established molecular detection technologies in the field.

IL-4 Detection Reagent Lyophilization. Previously, we reported that HaloTag-based ternary Nluc immunoassays can be lyophilized into an all-in-one homogeneous format, including antibody conjugates, LgTrip polypeptide, and the luciferase substrate.[9] As our new direct chemical labeling method could also potentially enable assays for point-of-need applications, we examined whether our reagents were compatible with lyophilization. Using the previously described formulation methods, we found that Ab5-β10 and Ab6-β9 conjugates as well as LgTrip 5146 were readily incorporated into all-in-one assay reagent cakes (Figure S15). Further, the lyophilized homogeneous reagent was easily reconstituted in an aqueous buffer, resulting in a single-step, add-and-read assay for IL-4 detection in a variety of sample matrices.

Assay Prototyping for αGamma Interferon (IFNγ), SARS-CoV2 Receptor Binding Domain (RBD), and Troponin I. To further probe the generalizability of ternary Nluc chemical conjugation, we aimed to generate assays for a variety of clinically important analytes. Similar to our efforts with IL-4, we attempted to identify an optimal pair of antibody conjugates for the detection of human IFNγ analyte, an important immune response marker for *Mycobacterium tuberculosis*.[29] A panel of seven commercially available IFNγ-specific antibodies (Table S2) was conjugated to β9 and β10 peptide labels and analyzed as described in the Supporting Methods. Like with the IL-4 system, we were able to rapidly (<3 h; 30 min actual hands-on time) generate antibody conjugates and identify a pair of labeled antibodies (Ab2-β10 and Ab5-β9) with good signal and dynamic range (Figure S16), even without optimization.

Using established compatible pairs of antibodies, we sought to develop simple, homogeneous immunoassays for the detection of SARS-CoV-2 RBD and Troponin I. Both targets could be detected using the ternary Nluc system (Figure S17). The ease by which the assays were developed (~2 h) demonstrates the general utility of the system for rapid prototyping of new assays across a variety of analytes. It is worth noting that although the Troponin I assay worked, it was not very sensitive. This target would therefore be an excellent candidate for exploiting our assay to screen for better antibodies to provide more sensitive detection.

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CONCLUSIONS

We report here the development of a ternary Nluc-based labeling technology and methodology for direct chemical conjugation of bioluminescent reporter peptides onto antibodies and subsequent screening for an optimized pairing of antibodies for immunoassay detection. The single-step, one-pot labeling method allows antibody conjugates to be quickly prepared without buffer exchange or other wash steps and immediately screened for the best performing pair(s). The workflow speed and simplicity represent a significant advancement for developing rapid screening assays for antibodies and other binders as reagents for molecular detection assays.

In future work, our labeling technology could be readily adapted to label and screen affinity reagent libraries (e.g., antibodies, antibody fragments, nanobodies) for high-throughput identification of novel binders against analytes of interest using automation. Moreover, the repertoire of conjugates could be expanded to primary amine-containing targets beyond proteins (e.g., nucleic acids, lipids), due to the small tag size and simplicity of peptide label conjugation chemistry. Future work can also explore further chemical derivatization of the label molecules, including site-specific reactive handles to improve labeling control and efficiency, and fluorophores for more defined peptide-conjugate characterization, multiplexing, and broadened applications.

METHODS

Label Synthesis. β9 and β10 peptide labels were synthesized based on procedures described in the Supporting Information.

LgTrip Evolution. A genetic site-saturation library of individual LgTrip point mutants was generated using LgTrip 3546 as the template (Twist Biosciences). The library was screened in *E. coli* lysates with complementary dipeptide as previously described.[9] Hits were genetically recombined using standard cloning techniques and rescreened for improved brightness. LgTrip 5146 was purified as previously described.[9] Kinetic biochemical analyses of LgTrip are detailed in the Supporting Information.

IL-6 Antibody Labeling. When indicated, antibodies 5IL6 and 505E (Thermo Fisher) were exchanged into sodium bicarbonate (pH 8.5) using 7 kDa molecular weight cut-off (MWCO) 0.5 mL Zeba columns (Thermo Fisher). If required, antibodies were concentrated to 1 mg/mL (6.7 μM) using Amicon 50 kDa MWCO microcentrifugal filters (Millipore Sigma). Conjugation reactions were performed in either sodium bicarbonate (pH 8.5) or the PBS solutions antibodies were provided in. Ten-fold molar excess of label (67 μM) was added to antibodies and incubated with end-over-end mixing at ambient temperature for 1 h, unless otherwise indicated. 5IL6 and 505E antibodies were conjugated to β9 and β10, respectively. Labeling reactions were quenched by adding Tris buffer (pH 7.5) to a final concentration of 100 mM and incubating for 5 min. For conjugate cleanup, crude reaction mixtures were exchanged into PBS (pH 7.0) via two passes through Zeba desalting columns. Conjugates were stored in PBS with 40% glycerol at −20 °C.

IL-6 Assay. Optimal assay concentrations of 5IL6-β9 and 505E-β10 were identified by titrating equimolar amounts of each antibody conjugate in PBS + 0.01% Blocker BSA (Thermo Fisher) (PBSB). The titration series was added 1:1 v/v to microtiter plate wells containing 2 μM LgTrip 5146 (saturating) ± 20 ng/mL recombinant human IL-6 analyte (R&D Systems) prepared in Roswell Park Memorial Institute (RPMI) cell culture media (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (HyClone). After incubation at ambient temperature for 1.5 h, a saturating, 30-fold dilution of the substrate (Nano-Glo Live Cell Substrate; Promega N205B) was added 1:1 v/v to assay plate wells. Luminescence was read on a GloMax Discover Luminometer (Promega) 10 min after substrate addition. The concentration of conjugates that gave the best assay dynamic range was utilized to assay a full IL-6 titration curve. A solution containing 125 ng/mL of 5IL6-β9 and 505E-β10 conjugates and 2 μM LgTrip 5146 was prepared in PBSB. The conjugate solution was mixed 1:1 v/v with an IL-6 titration prepared in cell culture media. After 1.5 h, the substrate was added and luminescence was read as described above.

IL-4 Antibody Screening. Six antibodies (see Table S1 for details) were conjugated in PBS (pH 7.0) at 1 mg/mL (6.7 μM) with a 10-fold molar excess of β9 or β10 label (67 μM). After 1 h at room temperature with end-over-end mixing, conjugation reactions were quenched by adding Tris buffer (pH 7.5) to a final concentration of 100 mM. Crude β9 and β10 antibody conjugate pairs were diluted to 500 ng/mL in PBSB containing 2 μM LgTrip 5146. Paired conjugate solutions were then mixed 1:1 v/v in 96-well assay plates with RPMI + 10% HI-FBS ± 100 ng/mL of recombinant human IL-4 analyte (PeproTech). After mixing for 30 s on an orbital shaker at 300 rpm, assay plates were incubated for 1 h at room temperature. Detection reagent with a 30-fold diluted substrate (saturating) was added 1:1 v/v with samples. Plates were briefly mixed on an orbital shaker and luminescence was read. Hits from the screen were washed via sequential passes through two Zeba columns and assayed against a titration of the IL-4 analyte using the same format as described above for IL-6.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.2c00306.

Supporting methods; supporting results (peptide linker analysis); and supporting figures (schemes for conjugation strategies, ternary Nluc complex formation and luminescence kinetics, SDS-PAGE analysis of antibody labeling reactions; impact of label linker length on labeling efficiency; impact of buffer on labeling efficiency; LgTrip 5146 protein sequence; LgTrip 5146 thermal stability analysis; LgTrip 5146 structure model; functional screen for anti-IL-4 antibody pairs; comparison between ternary Nluc and ELISA; compatibility of ternary Nluc reagents with lyophilization; additional models for screening antibody pairs; supporting tables; supporting synthetic chemistry schemes; supporting references; and an appendix summarizing mass analysis and yield for peptide conjugates) (PDF)

AUTHOR INFORMATION

Corresponding Authors

Virginia A. Kincaid − *Promega Corporation, Madison, Wisconsin 53711, United States;* orcid.org/0000-0002-0053-4577; Email: virginia.kincaid@promega.com

Lance P. Encell − *Promega Corporation, Madison, Wisconsin 53711, United States*; Email: lance.encell@promega.com

Melanie L. Dart − *Promega Corporation, Madison, Wisconsin 53711, United States;* orcid.org/0000-0001-5429-3695; Email: melanie.dart@promega.com

Authors

Hui Wang − *Promega Biosciences LLC, San Luis Obispo, California 93401, United States*

Casey A. Sondgeroth − *Promega Corporation, Madison, Wisconsin 53711, United States*

Emily A. Torio − *Promega Corporation, Madison, Wisconsin 53711, United States*

Valerie T. Ressler − *Promega Corporation, Madison, Wisconsin 53711, United States*

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Connor Fitzgerald − *Promega Biosciences LLC, San Luis Obispo, California 93401, United States*
Mary P. Hall − *Promega Corporation, Madison, Wisconsin 53711, United States*
Robin Hurst − *Promega Corporation, Madison, Wisconsin 53711, United States*
Monika G. Wood − *Promega Corporation, Madison, Wisconsin 53711, United States*
Julia K. Gilden − *Promega Corporation, Madison, Wisconsin 53711, United States*
Thomas A. Kirkland − *Promega Biosciences LLC, San Luis Obispo, California 93401, United States;* orcid.org/0000-0003-0859-5785
Dan Lazar − *Promega Corporation, Madison, Wisconsin 53711, United States*
Hsu Chia-Chang − *Promega Corporation, Madison, Wisconsin 53711, United States*
Thomas Machleidt − *Promega Corporation, Madison, Wisconsin 53711, United States*
Wenhui Zhou − *Promega Biosciences LLC, San Luis Obispo, California 93401, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.2c00306

Notes

The authors declare no competing financial interest.

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