# Converting your ELISA from horseradish peroxidase to alkaline phosphatase using NovaBright™ chemiluminescence detection reagents

## Introduction

Whether your current ELISA or other solid-phase immunoassay uses colorimetric, fluorimetric, or an alternative chemiluminescence readout, NovaBright™ chemiluminescence detection methods can help improve your assay performance, decrease the overall time to results, and reduce the quantity of antibody required. In this technical note, examples are provided to help guide conversion of your current assay to one with the features and benefits of Life Technologies NovaBright™ chemiluminescence detection reagents: DynaLight® Substrate with RapidGlow® Enhancer formulation.

An established ELISA protocol for the detection of human IL-10 using horseradish peroxidase (HRP)/colorimetric (tetramethylbenzidine (TMB)) detection was used as a model system for conversion to alkaline phosphatase (AP)/chemiluminescence detection using DynaLight® Substrate with RapidGlow® Enhancer. This technical note will be most useful if you already have an optimized assay in place in which both the capture and detection antibodies, as well as calibration standards, have been well characterized. However, even if assay development is just being started (including the choice of the best antibodies), this note should also be of use. The information provided here is for guidance only, and may not represent the specific or full spectrum of assay development activities required for conversion. These suggestions are applicable for conversion of HRP/colorimetric (TMB or other), HRP/luminescence (luminol or other), as well as AP/colorimetric (para-Nitrophenylphosphate (pNPP)), AP/fluorescent (4-methylumbelliferyl phosphate (MUP)), or other types of immunoassays.

### Materials and methods

| | |
|---|---|
| • Hu IL 10 antibody pair kit | Life Technologies, CHC1323 |
| • Antibody pair buffer kit | Life Technologies, CNB0011 |
| • Tris buffered saline 10X | Fisher Scientific, BP24711 |
| • Tween® 20 Solution | Sigma, P9416 |
| • Bovine serum albumin | Sigma, A3059 |
| • Clear 96-well microplates | Costar, 9018 |
| • White 96-well microplates | Costar, 3922 |
| • Black 96-well microplates | Costar, 3924 |
| • Alkaline phosphatase–conjugated streptavidin | Jackson ImmunoResearch, 016-050-084 |
| • DynaLight® Substrate with RapidGlow® Enhancer | Life Technologies, 4475406 |

Microplate luminometer, for example:

- Berthold Orion II luminometer
- Molecular Devices® SpectraMax® M5
- Thermo Scientific® Luminoskan Ascent

The items listed in Table 1 are recommended reading prior to beginning your assay conversion or new assay development.

**Table 1. Recommended reading.**

| Life Technologies Publication No. | Title | Comments |
|---|---|---|
| MAN0007146 | DynaLight® Substrate with RapidGlow® Enhancer and DynaLight® Trigger Solution | Guidance for performing immunoassays using our DynaLight® Substrate with RapidGlow® Enhancer reagent. |
| T9002 | ELISA-Light™ Immunoassay System | The ELISA-Light™ Immunoassay System product manual and protocols. |
| MAN0006706 | ELISA Technical Guide | General information and guidelines for using Novex® ELISA kits. Although much of this guide pertains to our colorimetric assays, many suggestions for good assay practices are also applicable to our NovaBright™ reagents. |

**General conversion notes**

- Converting from AP/colorimetric, AP/fluorimetric, or other AP/chemiluminescence systems based on AP-conjugated secondary antibody or AP-conjugated streptavidin:
  - If you are already using an AP-conjugated reagent for your immunoassay, the same AP conjugate can be used in your new assay. Conversion to NovaBright™ chemiluminescence detection reagents will still require re-optimization of the assay, because incubation times and the quantity of detection antibody(s) required may need to be reduced. Attempting to convert only the detection reagent in your original assay will likely result in high background and thus degrade the overall performance of the assay.
- Converting from HRP/colorimetric or HRP/chemiluminescence systems based on HRP-conjugated secondary antibody or HRP-conjugated streptavidin:
  - In the example provided in this technical note, the detection antibody used is biotinylated. The assay is converted to AP detection by swapping the HRP-conjugated streptavidin for AP-conjugated streptavidin. If you are using an HRP-conjugated secondary detection antibody, the analogous AP-conjugated secondary detection antibody should be used. If you are using direct-labeled detection antibody, the direct AP-conjugate will need to be purchased or prepared.

**Choosing a blocking reagent**

Standard ELISA blocking reagents are recommended; however, reagents should be checked to ensure that they do not contain contaminating AP. An example of a typical blocking buffer is Tris-buffered saline (pH 7.4), 1% BSA, and 0.05% Tween® 20 solution. Other proteins and detergents may be substituted according to your needs. Concentrations may need to be optimized for sufficient blocking based on your specific system. Note that phosphate-containing buffers should be avoided when performing alkaline phosphatase enzyme assays.

**Choice of microplate**

Solid black or solid white high-bind polystyrene plates are recommended. Black plates yield overall lower relative light units (RLUs) but offer the advantage of having little or no well-to-well light bleed-through. This feature is advantageous when calibrators or samples containing high antigen concentrations are being used. White plates provide higher RLU values and can provide improved low-end sensitivity for some assays. If you choose a solid white plate, note that signals from samples with very high chemiluminescence may bleed through to adjacent wells causing falsely elevated signals in wells with less concentrated samples, thus affecting both coefficients of variation (CVs) and final results. This potential effect should be considered when constructing your plate layout. Because these bleed-through problems are compounded by clear polystyrene plates, they are not compatible with chemiluminescence detection.

**Optimization of wash steps**

Standard ELISA wash protocols are recommended. Typically, 3 wash steps with Tris-buffered saline (pH 7.4), 0.05% Tween® 20 solution will be sufficient. Phosphate-containing buffers should be avoided when performing alkaline phosphatase enzyme assays.

# Converting your protocol

**General overview of optimization steps**

There are many factors that contribute to the performance of an ELISA. These include capture antibody concentration, detection antibody concentration, and secondary detection antibody concentration for indirect assays, as well as incubation times and temperatures. In many instances, it is possible to optimize 2 or more components at once in a checkerboard or grid titration. Note that NovaBright™ chemiluminescent substrates are extremely sensitive and may require broad titrations of your antibody reagents to determine optimal concentrations.

**Preparing your microplate**

This process assumes that you have already chosen appropriate coating and blocking buffers for your system. Starting antibody concentrations will vary depending on the type and purity of the antibody. The ideal way to determine optimal reagent concentrations is by performing a grid experiment using the layout in Figure 1 as a guide.

Chemiluminescence assays typically require less reagent; consequently, starting with your current concentrations is a good place to begin and then proceeding to dilute from that point. For example, if your current capture antibody concentration is 1 µg/mL then diluting 1:2 and 1:4 would result in testing 1, 0.5, and 0.25 µg/mL capture antibody concentrations. Similarly, start with your current concentration of detector antibody and dilute 1:4, 1:8, and 1:16. Knowledge of your assay system will guide you in determining which concentration ranges make the most sense for your titrations.

**Optimization of secondary detection format**

If your assay is an indirect method using a labeled secondary detector reagent, begin by keeping the concentration of the capture antibody constant. If necessary, you can titrate the capture antibody in a separate experiment. The most likely formats for this example are:

1. Detector = biotinylated anti-analyte antibody; secondary detector = streptavidin-conjugated enzyme (HRP or AP).

2. Detector = anti-analyte antibody; secondary detector = enzyme-labeled anti–species IgG antibody.

Note that your titration may need to be much broader than described in the example.

Choose concentrations of your standard that will interrogate the low-end sensitivity and measurement range of your assay. Starting with your lowest, mid-range, and highest standard curve calibrators is a good place to begin. NovaBright™ substrates typically improve sensitivity and expand dynamic range, so a broader range of analyte concentrations should be tested in subsequent experiments.

**Reagent titration example**

The following is an example of assay development data obtained when converting an IL-10 ELISA from HRP/colorimetric detection to AP/chemiluminescence detection. The assay employs indirect immunodetection based on using a biotin-labeled detector antibody and streptavidin-labeled alkaline phosphatase (SA-AP). Note that the assay was conducted as described in Figure 2. Table 2 shows the mean relative light units (RLUs) of duplicates.

**Figure 1. Layout of 96-well microplate used to determine optimal reagent concentrations for a primary detection assay.** In this example, the detector antibody is directly conjugated with AP. 1X refers to the concentration of reagents in your current assay.

| Standard concentration | 1X detector antibody | | | | | | 1:4 detector antibody | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 1X Capture | 1X Capture | 1:2 Capture | 1:2 Capture | 1:4 Capture | 1:4 Capture | 1X Capture | 1X Capture | 1:2 Capture | 1:2 Capture | 1:4 Capture | 1:4 Capture |
| A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low |
| C | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med |
| D | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi |
| E | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi |
| F | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med |
| G | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low |
| H | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1:8 detector antibody | | | | | | 1:16 detector antibody | | | | | |

**Figure 2. Layout of 96-well microplate used to determine optimal reagent concentrations for a secondary detection assay.** In this example, a secondary detector reagent conjugated with AP is used to reveal the primary detector antibody. 1X refers to the concentration of reagents in your current assay.

| Standard concentration | 1X secondary detector reagent | | | | | | 1:4 secondary detector reagent | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 1X Detector | 1X Detector | 1:4 Detector | 1:4 Detector | 1:8 Detector | 1:8 Detector | 1X Detector | 1X Detector | 1:4 Detector | 1:4 Detector | 1:8 Detector | 1:8 Detector |
| A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| B | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low |
| C | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med |
| D | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi |
| E | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi | Hi |
| F | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med | Med |
| G | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low | Low |
| H | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1:8 secondary detector reagent | | | | | | 1:16 secondary detector reagent | | | | | |

**Table 2. Data variability across reagent titration ranges.** Values are relative light units (RLUs) or ratios of RLUs, as indicated. The optimal results, as determined by signal-to-noise ratio (S/N), are highlighted in green.

| 1 µg/mL capture antibody | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/125,000 SA-AP | | | 1/62,500 SA-AP | | | 1/31,250 SA-AP | | | 1/15,125 SA-AP | | |
| IL-10 pg/mL | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector |
| 0 | 990 | 1442 | 2290 | 1944 | 2506 | 3863 | 2378 | 3683 | 5855 | 4386 | 5789 | 9056 |
| 5 | 10019 | 13165 | 16565 | 20021 | 23740 | 25743 | 25203 | 33610 | 39494 | 40553 | 49381 | 53038 |
| 50 | 87582 | 107909 | 129974 | 161376 | 197041 | 199823 | 209957 | 266551 | 295641 | 305856 | 348849 | 372350 |
| 5000 | 2625646 | 3441380 | 3885684 | 5556315 | 6463156 | 6837755 | 8055447 | 9783058 | 10498981 | 11283973 | 12025342 | 12099105 |
| S/N (low target) | 10.1 | 9.1 | 7.2 | 10.3 | 9.5 | 6.7 | 10.6 | 9.1 | 6.7 | 9.2 | 8.5 | 5.9 |
| S/N (high target) | 2654 | 2387 | 1697 | 2859 | 2579 | 1770 | 3387 | 2656 | 1793 | 2573 | 2077 | 1336 |

| 0.5 µg/mL capture antibody | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/125,000 SA-AP | | | 1/62,500 SA-AP | | | 1/31,250 SA-AP | | | 1/15,125 SA-AP | | |
| IL-10 pg/mL | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector | 0.04 µg/mL Detector | 0.08 µg/mL Detector | 0.16 µg/mL Detector |
| 0 | 629 | 867 | 1279 | 1034 | 1185 | 1583 | 2540 | 2766 | 3286 | 2523 | 2786 | 3960 |
| 5 | 3413 | 4360 | 4983 | 5020 | 6282 | 7093 | 9845 | 11433 | 13404 | 11286 | 14159 | 13942 |
| 50 | 25396 | 35592 | 38644 | 40207 | 49188 | 42057 | 70834 | 92720 | 102884 | 85889 | 103577 | 93536 |
| 5000 | 1014634 | 1393509 | 1569777 | 1700449 | 2166507 | 1949490 | 2796015 | 4247601 | 4386797 | 3955658 | 4972695 | 5286473 |
| S/N (low target) | 5.4 | 5.0 | 3.9 | 4.9 | 5.3 | 4.5 | 3.9 | 4.1 | 4.1 | 4.5 | 5.1 | 3.5 |
| S/N (high target) | 1613 | 1608 | 1228 | 1645 | 1829 | 1232 | 1101 | 1536 | 1335 | 1568 | 1785 | 1335 |

**In the IL-10 example given above, the optimal assay conditions based on S/N ratios are:**

| | |
|---|---|
| Capture Ab | 1 µg/mL |
| Biotinylated detector Ab | 0.04 µg/mL |
| Secondary SA-AP detector | 1/31,250 |

**For comparison, the optimal reagent concentrations for the IL-10 colorimetric assay are:**

| | |
|---|---|
| Capture Ab | 1 µg/mL |
| Biotinylated detector Ab | 0.16 µg/mL |
| Secondary SA-HRP detector | 1/2,500 |

### Analyzing your early assay development results

Check for strong positive signal versus low background noise, and then compare signal-to-noise ratios at low and high concentrations of your target to choose the best conditions for additional testing. Ideally, your titration ranges should capture your optimal reagent concentrations. Very low reagent concentrations will yield low positive signal, whereas very high concentrations will yield high background, both resulting in poor signal-to-noise ratios. If the optimal set of conditions has not been captured, you can repeat the grid experiments with expanded titration ranges. Careful analysis of results may help you identify which reagents need further titration. See the troubleshooting guide (Table 4) for more information.

### Choosing assay parameters

NovaBright™ chemiluminescent substrates provide improved sensitivity and dynamic range, while permitting a decrease in both assay time and quantities of detection reagents used. You may choose assay conditions that take advantage of all these improvements, or you may want to optimize for a particular feature. For example, with NovaBright™ DynaLight® Substrate, you may see up to 3 logs of sensitivity improvement, depending on your assay. If this level of detection range or sensitivity is not required for your analyte, you may be willing to sacrifice some sensitivity to decrease reagent usage by several-fold. Or, you may choose to optimize for decreased total assay time, with the goal of achieving equivalent or better results compared to the colorimetric assay. This flexibility allows you to tailor your assay to meet your specific needs.

### Optimizing for sensitivity

Chemiluminescent substrates are far more sensitive than colorimetric substrates, once optimized. Simply substituting AP/DynaLight® for HRP/TMB, for example, will likely result in saturated RLU readings. You should choose conditions that provide the greatest signal-to-noise ratio between a zero calibrator and a low sample (Figure 3), and verify your assay sensitivity according to your laboratory's preferred procedures, or refer to guideline EP17-A2 from the Clinical and Laboratory Standards Institute (CLSI) [1].

You can achieve optimal sensitivity and potentially decrease incubation times by titrating the concentrations of your capture, detector, and enzyme reagents.

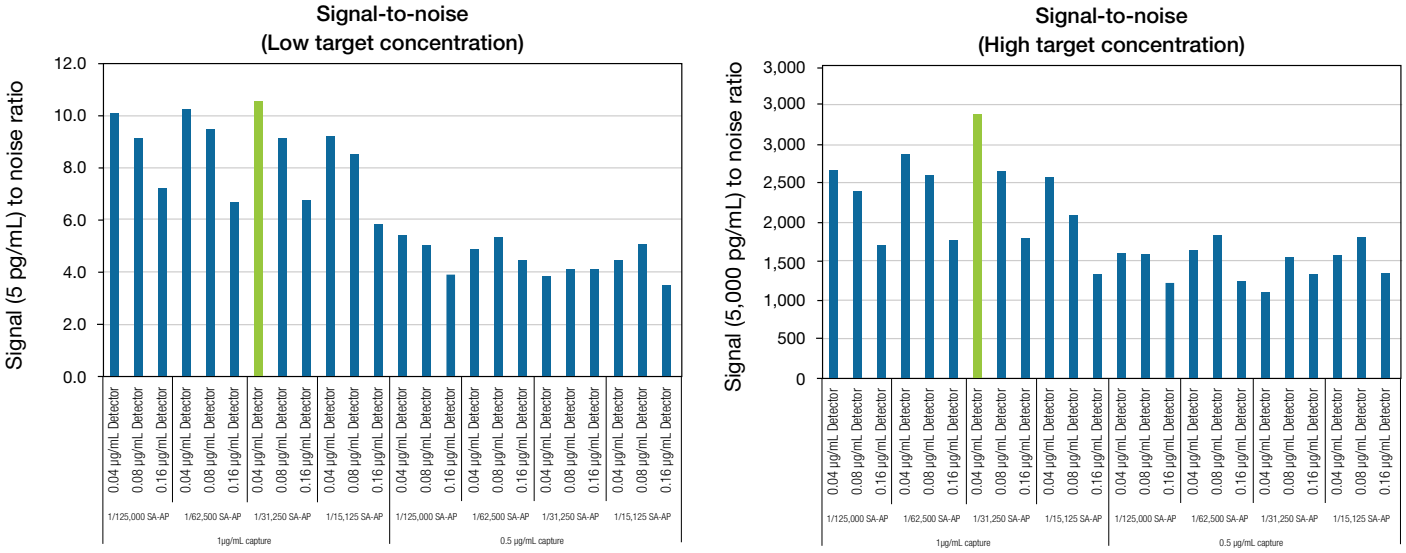


**Figure 3. Signal-to-noise ratios (S/N) for 5 pg/mL and 5,000 pg/mL IL-10 target, plotted across titration ranges.** Data shown in Table 2 are plotted as Signal/Noise across the SA-AP titration range. The green bars correspond to data shown in green in Table 2. This assay condition shows the best performance in S/N and would be used as a starting point to further fine-tune the assay conditions in a second round of titrations to define optimal conditions. Signal-to-noise (S/N) = RLU (lowest or highest control)/RLU (zero).

**Table 3. Colorimetric and NovaBright™ chemiluminescence assay conditions and outcomes compared.** Sensitivity: Conditions for optimal sensitivity were determined from the reagent titrations shown in Table 2 and Figure 3. Reagent preservation: The concentration of capture antibody was decreased by 2-fold to demonstrate reagent preservation. Note that the total assay time is still shorter than the colorimetric protocol, despite an increase in the sample and detector antibody incubation times relative to the other chemiluminescence assays. Minimization of incubation times: To minimize total assay time, all incubations were reduced to 10 minutes. Detector antibody concentration is still lower than in the colorimetric protocol, despite an increase relative to the optimal concentration for sensitivity.

| | | NovaBright™ chemiluminescence, optimized for | | |
|---|---|---|---|---|
| Assay conditions | Colorimetric | Sensitivity | Reagent preservation | Minimization of incubation times |
| Capture µg/mL | 1 | 1 | 0.5 | 1 |
| Detector µg/mL | 0.16 | 0.04 | 0.04 | 0.08 |
| SA-enzyme dilution | 1/2500 | 1/31,250 | 1/31,250 | 1/31,250 |
| Total incubation (minutes) | 180 | 70 | 100 | 30 |
| *Sample & detector Ab* | *120* | *30* | *60* | *10* |
| *Streptavidin-enzyme* | *30* | *30* | *30* | *10* |
| *Substrate* | *30* | *10* | *10* | *10* |
| *Limit of blank (pg/mL) | 2.570 | 0.090 | 0.209 | 0.313 |
| Dynamic range | 3 logs | 4 logs | 4 logs | 4 logs |

*Limit of blank = Corresponding concentration of mean blank RLU measurements + 2 standard deviations of blank RLU measurements.

## Optimizing for reagent preservation

Chemiluminescent substrates can often deliver sub-pg/mL sensitivity, depending on the quality of antibodies and other assay conditions. This level of sensitivity may not be required, depending on the analyte and application of the assay being converted. If this is the case, you may opt to sacrifice some sensitivity so that you can conserve your precious or expensive antibody reagents. In this scenario, you can titrate your reagents to very low concentrations while maintaining excellent sensitivity.

## Optimizing for time to results

Colorimetric assays can be time-consuming, due to multiple incubation steps of an hour or more each that are required to achieve desired levels of sensitivity. In general, the superior sensitivity of chemiluminescence allows for shorter incubation times. If assay speed is important, you may find that you can decrease your incubation times significantly, while still achieving sensitivity that meets your needs. In the example in Table 3 and Figure 4, the assay time has been reduced to 30 minutes while maintaining reduced reagent use. Note that assay performance using these conditions is still superior to that of the colorimetric assay.

Table 3 provides a comparison of the original optimized colorimetric IL-10 assay conditions and performance compared to the chemiluminescence assay optimized for sensitivity, reagent preservation, or minimal incubation times. Data gathered using conditions in Table 3 are plotted in Figure 4.

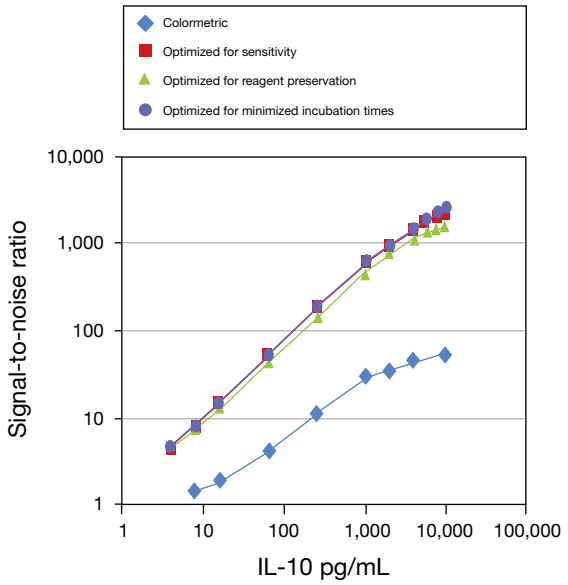


**Figure 4. Superior dynamic range and signal-to-noise ratios are achieved with DynaLight® Substrate.**

## Troubleshooting

Table 4 offers some suggestions for problems that may arise during your assay development when converting your ELISA from another mode of detection to AP/chemiluminescence detection.

## Additional testing

You should verify the performance of the chemiluminescence assay once you have determined your optimal assay conditions. You may need to make adjustments to your standard curve to take advantage of any gains in sensitivity and dynamic range. You should also check the accuracy of the assay by running characterized samples, if possible. Finally, you should perform an analytical evaluation by determining the limit of detection, precision, and any other studies that are appropriate for your assay and application.

## Summary

There are many advantages to converting your colorimetric ELISA to a NovaBright™ chemiluminescence ELISA. Superior sensitivity and dynamic range, combined with the flexibility to help decrease reagent usage and time to results, allow you to tailor your assay to meet your specific needs.

References

1. Pierson-Perry JF, chairholder (2012) *Evaluation of detection capability for clinical laboratory measurement procedures; approved guideline*. 2nd ed. Wayne (PA): Clinical and Laboratory Standards Institute. CLSI document EP17-A2.

**Table 4. Troubleshooting your ELISA conversion.**

| Problem | Possible cause |
|---|---|
| High background | •Too much detector and/or capture antibody<br>•Insufficient washing<br>•Insufficient blocking<br>•Cross-reacting substance in sample matrix<br>•Buffer contamination<br>•Incubation time too long |
| No signal | •Not enough detector and/or capture antibody<br>•Capture antibody not bound<br>•Degradation of standard or sample<br>•Interfering substance in sample matrix<br>•Buffer contamination<br>•Incubation time too short |
| High signal across plate | •Insufficient washing<br>•Too much detector<br>•Buffer contamination<br>•Incubation time too long |
| Poor replication | •Insufficient washing<br>•Uneven plate coating<br>•Uneven reagent addition |

## Ordering information

| Product | Quantity | Cat. No. |
|---|---|---|
| DynaLight® Trigger Solution | 100 mL | 4475403 |
| DynaLight® Trigger Solution | 1 L | 4475409 |
| DynaLight® Substrate with RapidGlow® Enhancer | 100 mL | 4475406 |
| DynaLight® Substrate with RapidGlow® Enhancer | 1 L | 4475410 |
| IL 10 Human Antibody Pair Kit | 10 Plates | CHC1323 |
| Buffer Kit for Antibody Pairs | 10 Plates | CNB0011 |

View all products online at **lifetechnologies.com/DynaLight**

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