INSTRUCTIONS

Thermo SCIENTIFIC

QuantaRed™ Enhanced Chemifluorescent HRP Substrate

15159

2063.1

Number	Description
15159	**QuantaRed Enhanced Chemifluorescent HRP Substrate,** sufficient reagents for 10 × 96-well assays
	Contents:
	QuantaRed ADHP Concentrate, 1 ml
	QuantaRed Enhancer Solution, 50 ml
	QuantaRed Stable Peroxide Solution, 50 ml
	QuantaRed Stop Solution, 10 ml
	Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific QuantaRed Enhanced Chemifluorescent HRP (horseradish peroxidase) Substrate contains chemical enhancers to increase the fluorescent yield and sensitivity of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) chemifluorescence. ADHP is a non-fluorescent compound that reacts with HRP to produce resorufin, a soluble, highly fluorescent reaction product with excitation/emission maxima at ~570/585 nm. QuantaRed Enhanced HRP Substrate has sensitivity comparable to enhanced chemiluminescence and is the most sensitive chemifluorescent substrate available for detecting HRP in ELISAs. Unlike chemiluminescent reactions, the QuantaRed Substrate reaction can be stopped and the fluorescent signal remains stable for several hours. The long wavelength emission spectrum of resorufin minimizes interference from low-wavelength autofluorescence (blue and green) that exists in many biological samples.

The QuantaRed Substrate is detected using the appropriate excitation and emission settings (Figure 1). Quantitation does not require filters that precisely match the excitation/emission maxima; however, a non-overlapping filter set with a bandpass that includes the excitation/emission spectra is required. Wavelengths at 530-575 nm for excitation and 585-630 nm for emission can be used for detection. Also, the reaction product can be measured at 576 ± 5 nm on a colorimetric plate reader.

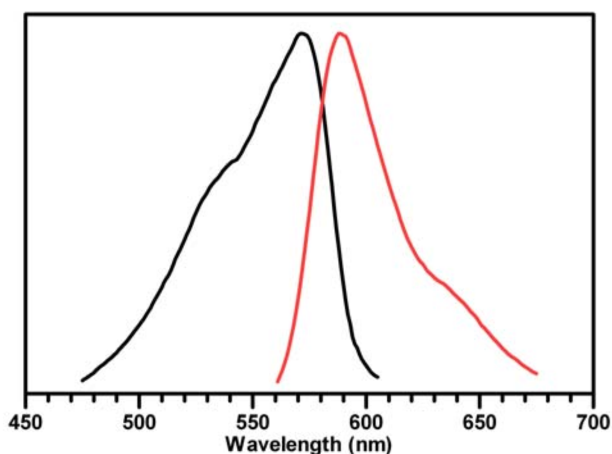

Figure 1. Normalized absorption (left) and emission (right) spectra of the Thermo Scientific QuantaRed Enhanced Chemifluorescent HRP Substrate.

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Important Product Information

- Instrumentation-specific settings will affect the performance of the QuantaRed Substrate. High gain settings will increase signal intensity. Increasing the photomultiplier tube voltage will also increase signal intensity. Increase or decrease the bandpass range of the excitation and/or emission filter to achieve signal intensity requirements.
- Fluorometric units are typically defined as relative fluorescence units (RFU) because the integrated signal is dependent on instrument settings. Consult the fluorometer's instructions for specific instrument capabilities and settings.
- Fluorometric assays typically use black or white opaque microplates. White plates typically offer greater signal detection and higher background than black plates. A variety of other plate types, such as gray or opaque plates with transparent bottoms, are also useful with this substrate.
- To avoid cross-contamination of the substrate bottles when preparing the working solution, use a new pipette tip for each component. Be careful not to contaminate the substrate with peroxidase when adding the working solution into the wells.
- If high background is produced, optimize the assay components, such as antibody, conjugate and blocking buffer. Opaque black plates often result in less background than opaque white plates.
- Shaking the plate briefly after adding the QuantaRed Stop Solution is not required but may decrease the standard deviation because the well contents become more homogenous. Furthermore, increasing the flash count or integration time on the fluorometer might improve precision of replicates.
- QuantaRed Substrate Solution and Enhancer Solution are air- and light-sensitive. Close container tightly after each use.
- Equilibrate all reagents to room temperature before use. Temperature can affect the reaction kinetics.
- Do not use Tween®-20 Detergent for washes before adding the substrate working solution, which can increase background. If Tween-20 Detergent is used for washing, perform 2-3 additional washes with a suitable buffer that does not contain detergent.

Example Microplate Procedure for Detecting Horseradish Peroxidase

Note: For best results, optimize all variables for each specific system. Suggested antibody ranges are as follows:

- Capture antibody at 1-10 µg/ml
- Detection antibody at 0.05-1 µg/ml
- HRP conjugate at 0.1-0.2 µg/ml

1. Use standard ELISA procedures to coat, block and wash microplate wells as required.

   Note: For prolonged incubation times or incubation at elevated temperatures, cover the plate with sealing tape (Product No. 15036) to prevent sample evaporation.
2. Mix 50 parts QuantaRed Enhancer Solution with 50 parts QuantaRed Stable Peroxide and 1 part of the QuantaRed ADHP Concentrate. Example: mix 5 ml QuantaRed Enhancer Solution with 5 ml QuantaRed Stable Peroxide and 100 µl of the QuantaRed ADHP Concentrate. Use working solution within 30 minutes of preparation.
3. Add 100 µl of QuantaRed Working Solution to each microplate well and incubate for 1-15 minutes at room temperature.
4. Stop peroxidase activity by adding 10 µl of QuantaRed Stop Solution and shake plate for 10-30 seconds. The enzymatic activity is immediately stopped and incubation is not required.

   Note: Peroxidase activity will generate a strong pink signal. Preliminary plate evaluations may be performed without stopping because the substrate does not photobleach and assay progress is not negatively affected by exposure to light. At high peroxidase concentrations or with prolonged reaction times, the fluorescent product may self-quench, which is observed as continued color development without a change in fluorescence intensity. Optimization of the detection antibody or HRP conjugate might be required to determine the linear range.
5. Measure relative fluorescence units (RFU) of each well. The excitation and emission maxima for QuantaRed Substrate are 570 nm and 585 nm, respectively. An excitation of 530-575 nm and emission of 585-630 nm also may be used. Alternatively, the reaction product can be measured at 576 ± 5 nm on a colorimetric plate reader.

Troubleshooting

Problem	Possible Cause	Solution
High background	HRP concentration is too high	Optimize HRP conjugate concentration
	Blocking was insufficient	Optimize blocking time or temperature (e.g., block for at least 1 hour at RT or overnight at 4°C)
		Optimize blocking buffer – the best blocking buffer is system-dependent
		Increase blocking buffer protein concentration
	Cross-reactivity of capture and detection antibodies	Try a different antibody pair
	Insufficient washing	Add additional washes after incubation with HRP conjugate
	Tween-20 was added to wash buffer	Wash wells with buffer that does not contain detergent before adding the substrate working solution
Low signal	Low amount of target protein	Add more sample
	Capture or detection antibody concentrations are not optimal	Optimize antibody concentrations
	Excitation and emission wavelengths are not optimal	Use instrumentation settings or filter sets that are closer to the substrate's excitation and emission maxima
	Instrument settings (i.e., gain, voltage) are not optimal	Refer to instrument instructions or contact instrument manufacturer

Related Products

37538 **StartingBlock™ Blocking Buffer in PBS**

37542 **StartingBlock Blocking Buffer and in TBS**

N502 **ELISA Blocker Blocking Buffer**

21130 **High-Sensitivity Streptavidin HRP**

References

1. Crowther, J. R. (1995). ELISA Theory and Practice: Humana Press. Totwa, NJ.
2. Brotea, P.G., (1988). Fluorometric determination of hydrogen peroxide using resorufin and peroxidase. Microchemical Journal 37, 368-376

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