

Homogeneous Time-Resolved Fluorescence

Part 2: Ratio & data reduction

Abstract

Homogeneous Time-resolved Fluorescence (HTRF®) assays are straightforward to perform. They do not require a separation or wash step. To reduce the potential interference from biological media or compounds left in the plate during detection, we have patented an HTRF ratiometric data reduction. Further, the ratiometric value allows the end user to look at multiple aspects of the data generated. Additional calculations and methods of data reduction will be described in this application note.

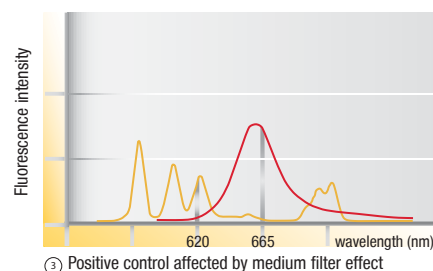
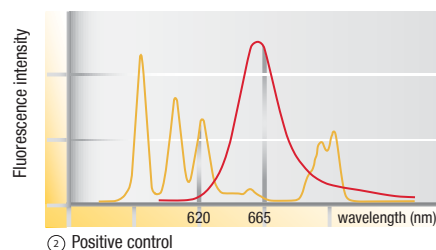
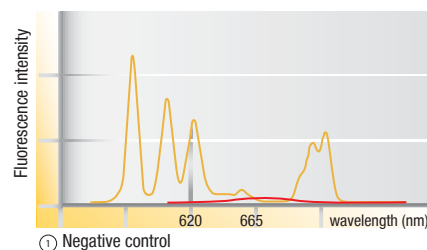
Ratiometric data reduction: A straightforward way to eliminate compound interference.

HTRF technology uses either Eu^{3+} or Tb^{2+} Cryptate as the donor fluorophore, and either XL665 or d2 as the acceptor fluorophore. For these fluorophores, we recommend to measure the fluorescence emission at 620 nm for the donor and at 665 nm for the acceptor.

The measurement of HTRF emissions at two different wavelengths (620 nm and 665 nm) allows for the ratiometric reduction of data. This feature of HTRF is extremely advantageous, particularly for reducing well-to-well variations that may arise in homogeneous assay formats where a separation or wash step is not performed. Compounds and/or media additives left in the plate have the potential to change the photophysical properties in a given sample and the degree to which this occurs may vary from sample to sample. By using the ratio of the donor and acceptor emission signals, it is possible to eliminate compounds that are simply interfering with detection.

Cisbio has developed and patented a ratiometric measurement that uses both the emission wavelength of the donor and acceptor (patent US 5,527,684 and foreign equivalents) to correct for well-to-well variability and signal quenching from assay components and media. Emissions at 620 nm (donor) are used as an internal reference while emissions at 665 nm (acceptor) are used as an indicator of the biological reaction being assessed.

	Channel A 665 nm (acceptor)	Channel B 620 nm (donor)	Ratio (A/B) x 10 ⁴	Mean ratio
①	1,459 1,392	35,178 35,547	415 405	410
②	10,416 10,615	34,331 34,612	3,009 2,986	2,998
③	4,620 4,197	14,952 14,213	3,090 2,953	3,022



Measurement of the total (positive control) and background (negative control) signal was carried out as well as the signal generated in the presence of a colored compound possessing quenching capabilities. As expected, the positive and negative controls show a clear difference in the absolute signal intensity at 665 nm and not at 620nm (see scheme previous page).

This corresponds to an appropriate change in the HTRF ratio for the two sample types. However, in the sample containing the colored compound, a similar decrease in both 620 nm and 665 nm signals is observed. Since the degree of sample quenching in both emissions is similar, the HTRF ratio remains relatively unchanged. This shows that the compound is non-specifically interfering with the total emission in the sample and demonstrates the benefit of using the ratiometric data reduction to eliminate interfering compounds in an assay.

If the ratio had not been calculated, the sample would be falsely identified as an inhibitor of the biological reaction being tested in the assay. This clearly demonstrates the usefulness of calculating the 665/620 ratio for each well.

What you must know about data reduction

Case study: standard curve in a competitive assay

Ratiometric data reflects the “raw” results

The ratio must be calculated for each well individually. The mean and standard deviation can then be worked out from replicates. The 10⁴ multiplying factor is introduced for easier data processing:

	Channel A 665 nm	Channel B 620 nm	Ratio (A/B) x 10 ⁴
Background	2,040	40,765	500
Std 0	45,999	41,442	11,100
Std 1	40,615	41,000	9,906
Std 2	29,212	41,732	7,000
Std 3	15,249	40,124	3,800
Std 4	6,258	39,124	1,600

$$\text{Ratio} = \frac{A_{665 \text{ nm}}}{B_{620 \text{ nm}}} \times 10^4$$

Delta Ratio reflects the “specific signal”

The delta ratio is obtained by subtracting the background from the signal of each positive point.

	Channel A 665 nm	Channel B 620 nm	Ratio (A/B) x 10 ⁴	Δ R
Background	2,040	40,765	500	
Std 0	45,999	41,442	11,100	10,599
Std 1	40,615	41,000	9,906	9,406
Std 2	29,212	41,732	7,000	6,499
Std 3	15,249	40,124	3,800	3,300
Std 4	6,258	39,124	1,600	1,099

$$\Delta R = \text{Ratio}_{\text{std}} - \text{Ratio}_{\text{background}} = \text{Signal} - \text{Background fluorescence}$$

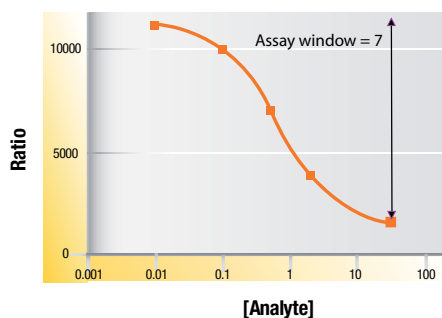
Assay window (Signal max/Signal min)

The window is obtained by dividing the maximum signal ratio value by the minimum signal ratio value.

	Channel A 665 nm	Channel B 620 nm	Ratio (A/B) x 10 ⁴	Assay window
Std 0	45,999	41,442	11,100	
Std 1	40,615	41,000	9,906	
Std 2	29,212	41,732	7,000	
Std 3	15,249	40,124	3,800	
Std 4	6,258	39,124	1,600	

$$\text{Assay window} = \text{Ratio}_{\text{assay max}} / \text{Ratio}_{\text{assay min}}$$

max = Std 0 min = Std 4



In depth use of the normalized signal

Delta F for inter-assay comparisons

Delta F is used for the comparison of day to day runs of the same assay. It reflects the signal to background of the assay. The negative control plays the role of an internal assay control.

Assay DAY 1	Channel A 665 nm	Channel B 620 nm	Ratio (A/B) x 10 ⁴	Δ F
Background	2,228	48,765	457	
[Molecule 1]	8,294	45,442	1,825	299%
[Molecule 2]	14,999	46,000	3,261	614%

Assay DAY 2	Channel A 665 nm	Channel B 620 nm	Ratio (A/B) x 10 ⁴	Δ F
Background	8,021	49,875	1,608	
[Molecule 1]	31,315	48,997	6,391	297%
[Molecule 2]	57,466	50,001	11,493	615%

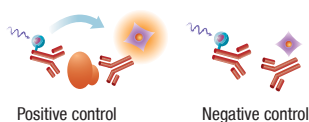
$$\Delta F = \frac{\text{Ratio}_{\text{sample}} - \text{Ratio}_{\text{background}}}{\text{Ratio}_{\text{background}}} \%$$

$$\frac{\text{Signal} - \text{Background}}{\text{Background}}$$

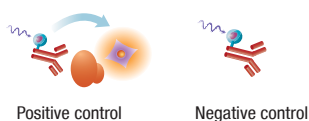
Determination of the negative control

Depending on the assay type, the negative ratio may be either the negative control of the assay for sandwich formats or the cryptate blank for direct binding partner assays (e.g. immunocompetitive assays).

Sandwich assays format



Competitive assay format



Delta F / Delta F max enables the comparison of two curves

This calculation is used for normalizing the signal in competitive assays.

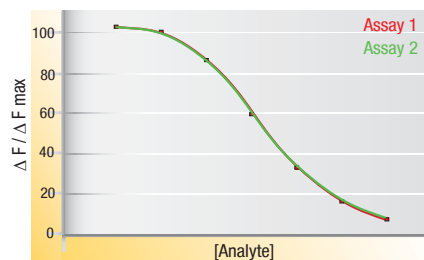
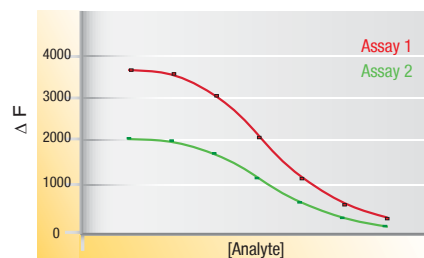
Assay 1	Channel A 665 nm	Channel B 620 nm	Ratio (A/B) x 10 ⁴	Δ F	Δ F / Δ F max
Background	2,040	40,765	500		
Std 0	45,999	41,442	11,100	2,118%	100%
Std 1	40,615	41,000	9,906	1,880%	89%
Std 2	29,212	41,732	7,000	1,299%	61%
Std 3	15,249	40,124	3,800	659%	31%
Std 4	6,258	39,124	1,600	220%	10%

Assay 2	Channel A 665 nm	Channel B 620 nm	Ratio (A/B) x 10 ⁴	Δ F	Δ F / Δ F max
Background	2,140	42,765	500		
Std 0	75,241	43,242	17,400	3,377%	100%
Std 1	69,319	45,100	15,370	2,971%	88%
Std 2	49,115	44,732	10,980	2,094%	62%
Std 3	25,098	43,124	5,820	1,063%	31%
Std 4	9,991	43,924	2,275	355%	10%

$$\frac{\Delta F (\text{sample})}{\Delta F \text{ max.}}$$

$$\Delta F \text{ max.}$$

max = std 0
sample = standard



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