

Application Note

Quench Correction for LANCE™ Time-Resolved Fluorescence Resonance Energy Transfer

INTRODUCTION

In LANCE™ TR-FRET assays the sample interference can be corrected from the time-resolved acceptor signal (A) based on the fact that the energy transfer signal is a direct function of the excited states of donors in the complex (i.e. the integral of donor emission within the chosen time-window, D). This traditional 'ratio measurement' is improved by subtracting both blank (B1) and donor cross-talk (c x D) values from the time-resolved integral of acceptor signal (A) before calculation of the ratio. The corrected values are defined as **blank-corrected normalized ratio** (R_n). Use of the equation requires a configuration plate (or wells) where the plate blank, donor-caused background and

normalization factor is defined for the particular assay. It should be noted that all measurements have to be made with identical instrumental parameters with the exception of filters (for donor and acceptor).

This application note will demonstrate LANCE Normalization with Wallac brand instruments.

There are two options available to perform the LANCE Normalization. LANCE Normalization is done either using the WIZARD software, which will guide you through the process or the Normalization samples are run among other samples in sample plate.

LANCE NORMALIZATION

Calculation of the blank-corrected normalized ratio requires three sets of samples to be run before the assay (LANCE Normalization wizard). LANCE normalization samples can be analyzed as a part of the assay under protocol editor as well. The assay plate, buffer, reagent concentrations and volumes should be the same as in the actual screening.

Check the instrument manuals for the number of replicates needed for the LANCE Normalization wizard. If normalization samples are run among the other samples using the protocol editor for sample definition, the number of replicates can be freely chosen.

1. Blank control. Non-colored pure assay buffer (Bl).
2. Cross-talk control. Donor-labeled reagent (e.g. Eu) in the assay buffer and assay concentration. This is used to calculate the cross-talk factor, c , expressed as the fraction of donor signal at the acceptor's measuring window.
3. High reference. Wells containing all the reagents required forming maximal energy transfer signal. The concentrations should be the same as in actual screening. The donor intensity, D_{max} is used to calculate the normalized ratio giving numeric values similar to the unquenched energy transfer intensity.

MEASUREMENT

Each well is measured with both donor (D) and acceptor (A) filters using identical time-windows. The following values will be needed for calculations:

Bl: The A measurement of the buffer wells (1) i.e. the plate background with acceptor's measuring window (e.g. at 665)

c: $(A_c - Bl)/D_c$, the cross-talk factor (c) is obtained from cross-talk control wells (2) giving the ratio by which the donor gives background at acceptor's measuring window (A-Bl) compared to donor signal at donor's measuring window (D)

D_{max} : The donor signal (D) in high reference wells (3). This value is used for normalization.

All samples measured with same parameters (D and A) are corrected with the following equation to give blank-corrected normalized ratio values (R_n):

$$R_n: (ET / D_s) \times D_{max} = [(A_s - Bl - c \times D_s) / D_s] \times D_{max}$$

Where:

ET is the energy transfer signal

A_s is the fluorescence intensity of the sample at acceptor's measuring window

D_s is the fluorescence intensity of the sample at donor's measuring window

Bl, c and **D_{max}** are obtained from the configuration

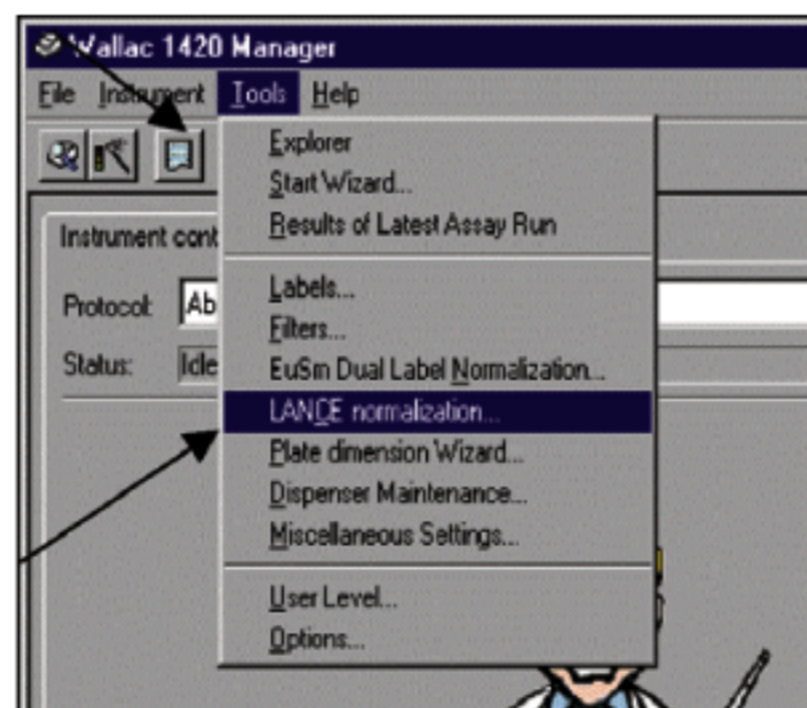
INSTRUCTIONS FOR LANCE NORMALIZATION WITH WALLAC BRAND PLATE READERS AND IMAGERS

VICTOR²_{TM}

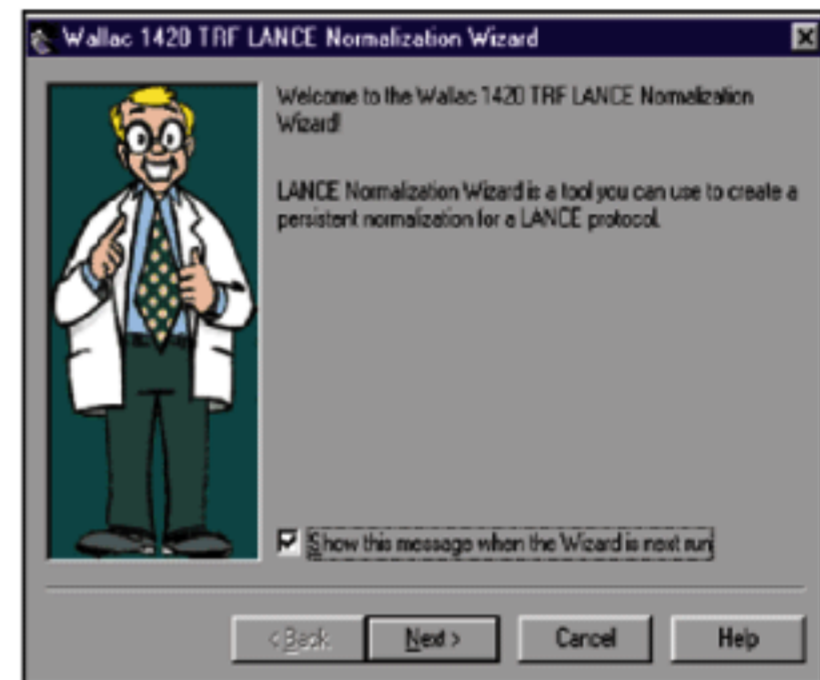
1. Define a new protocol which has the appropriate labels and plate.



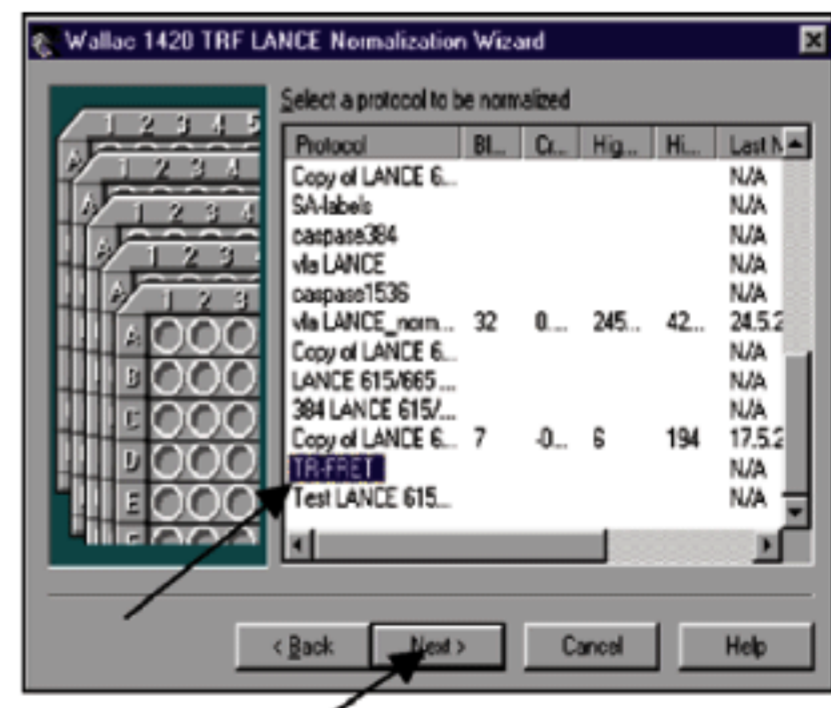
2. Start the LANCE Normalization WIZARD from the Tools menu using the manager function.



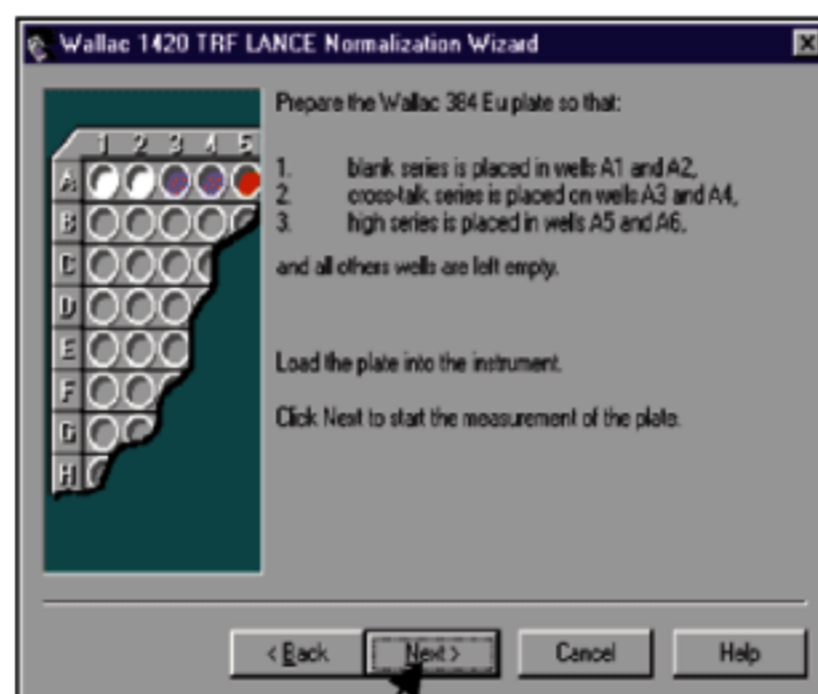
3. The software will start the LANCE Normalization Wizard and it will guide you through the process.



4. Select the appropriate protocol. In this table will you see whether the protocol has been normalized and the data was normalized. You will also see the information for blank, cross-talk, high cross-talk and high signals if the protocol has been normalized.

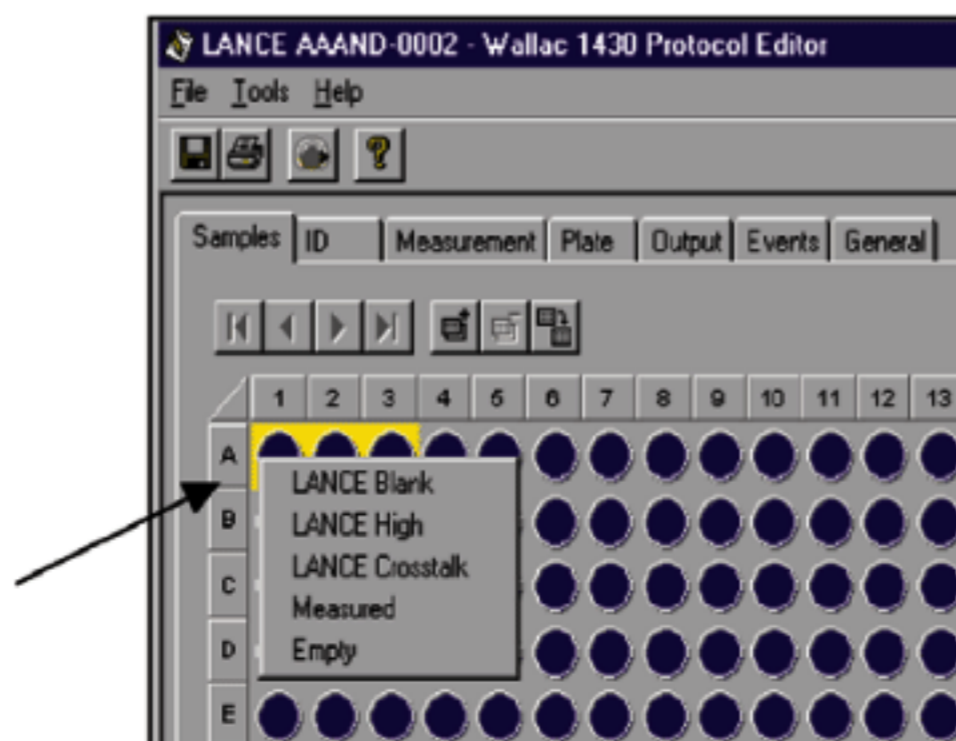


5. If you use the LANCE Normalization Wizard, the software will guide you to put blank samples (buffer) in wells A1 and A2, cross-talk samples (donor reagent in assay buffer, eg europium) in wells A3 and A4 and high samples (all the reagents needed to form the maximal energy transfer signal) in wells A5 and A6.



6. By clicking next-button the software will start the normalization procedure. Once it has been performed for the protocol it is saved as long as no changes are made for the protocol.

7. LANCE blank, cross-talk and high samples can be run among other samples as well. By using this option the number of replicates can be chosen. Software will calculate the normalized LANCE signal as in the LANCE Normalization Wizard.

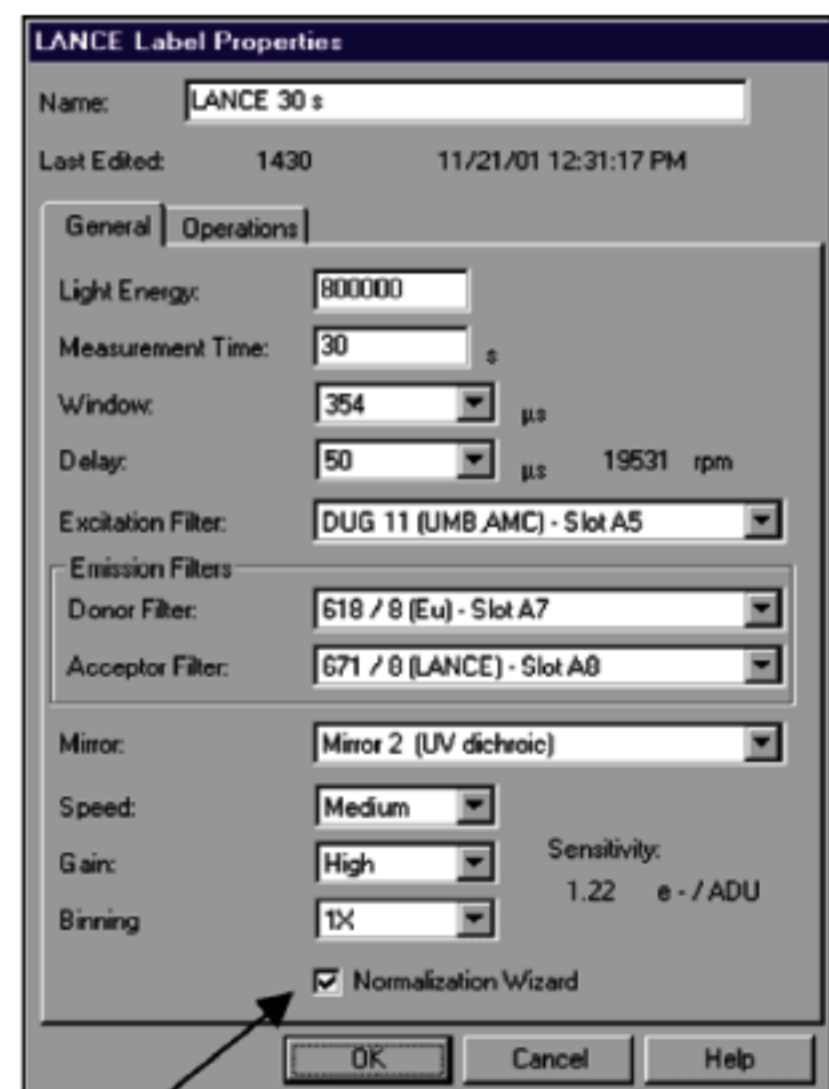


8. The result file will include the results for 615 nm and 665 nm signals as well as normalized 665 nm signals.

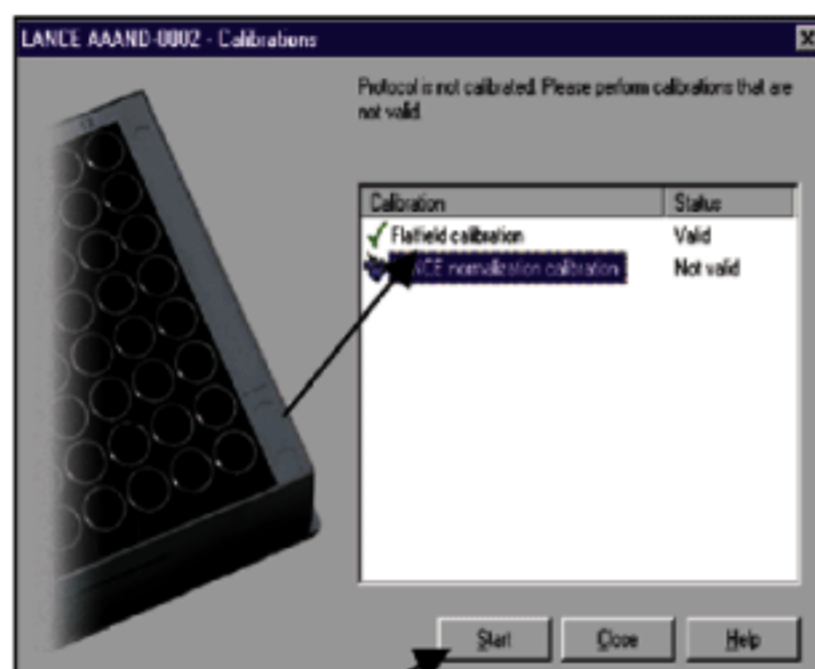
ViewLux™

1. LANCE Normalization can be performed with ViewLux as a part of the protocol calibrations or as in VICTOR², among other samples (see the picture 7).

2. If LANCE Normalization is used as a part of the calibrations, check the normalization wizard under label properties.

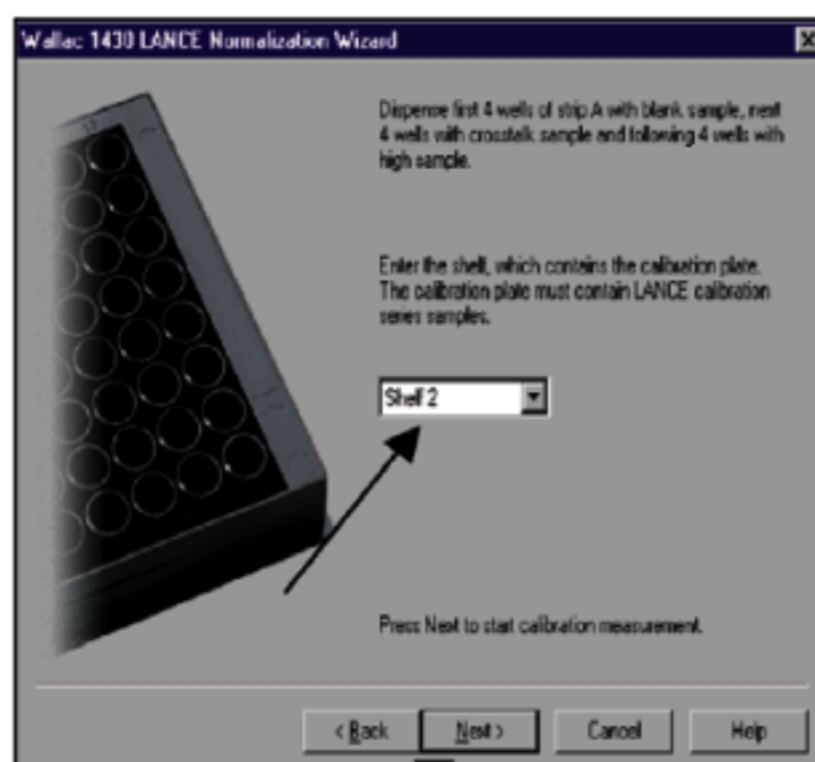


3. Save the protocol. The Protocol Editor will lead you to the calibrations function. Begin the flatfield calibration and the LANCE normalization. The software will guide you through the normalization.



4. ViewLux software requires you to put 4 replicates of blank, cross-talk and high samples in appropriate wells.

5. Start the normalization by choosing the shelf.



6. After the normalization the instrument will link the normalization data for your protocol as long as no changes have been made to the protocol.

EnVision™

LANCE Normalization is available also in EnVision. The normalization samples are defined under protocol.

MATERIALS AND METHODS

To test the efficiency of the quench correction, a model assay based on LANCE kinase assay (Figure 1) was set up.

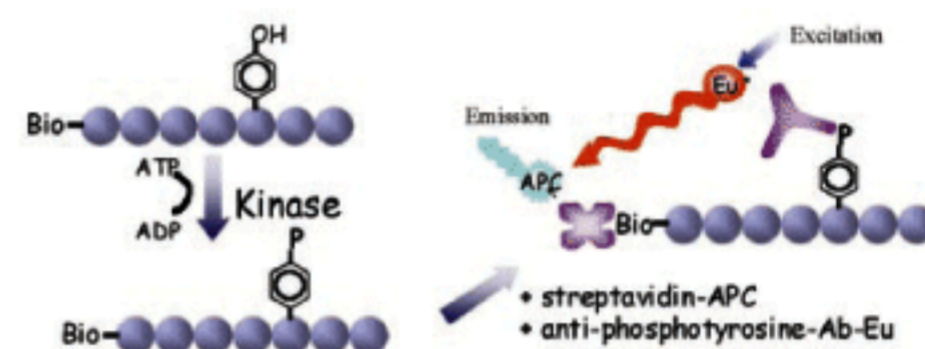


Figure 1. Principle of the LANCE kinase assay (Hemmilä and Ahola, 1997).

The LANCE normalization was performed according to the previous instructions.

The samples contained 50 μ l water (blank) or color dilution (Table 1), 50 μ l 50nM phosphorylated bio-peptide (Bachem H-1546), 100 μ l 8 μ g/ml streptavidin-APC and 50 μ l 4nM PT-66-Eu antibody. All dilutions were made on TSA-BSA (0.1%) buffer. The total volume of the reaction was 250 μ l. The mixture was incubated at RT for 15 minutes and both donor and acceptor signals were measured with VICTOR². After the measurement, aliquots of 75 and 150 μ l were transferred to fresh plates and measured again. Quench-corrected results were calculated using the equation above to get blank-corrected normalized ratio values (R_n) (Figures 2 and 3).

Table 1. The tested colors and their concentrations

Sample#	Color	Conc. (μ M)	Sample#	Color	Conc. (μ M)
1	H ₂ O		15	Methylene blue	4
2	Methylene orange	40	16	Methylene green	0.5
3	Methylene orange	150	17	Methylene green	3
4	Methylene red	75	18	Methylene green	50
5	Methylene red	300	19	Chicago sky blue	2
6	Tartrazine	50	20	Chicago sky blue	10
7	Tartrazine	200	21	Indigocarmine	5
8	Water blue	25	22	Indigocarmine	20
9	Water blue	100	23	Naphthol green	20
10	Cherry red	25	24	Naphthol green	100
11	Cherry red	100	25	Patent blue	5
12	Light green SF	50	26	Patent blue	16
13	Light green SF	100	27	Bromophenol blue	5
14	Methylene blue	0.5	28	Bromophenol blue	20

RESULTS

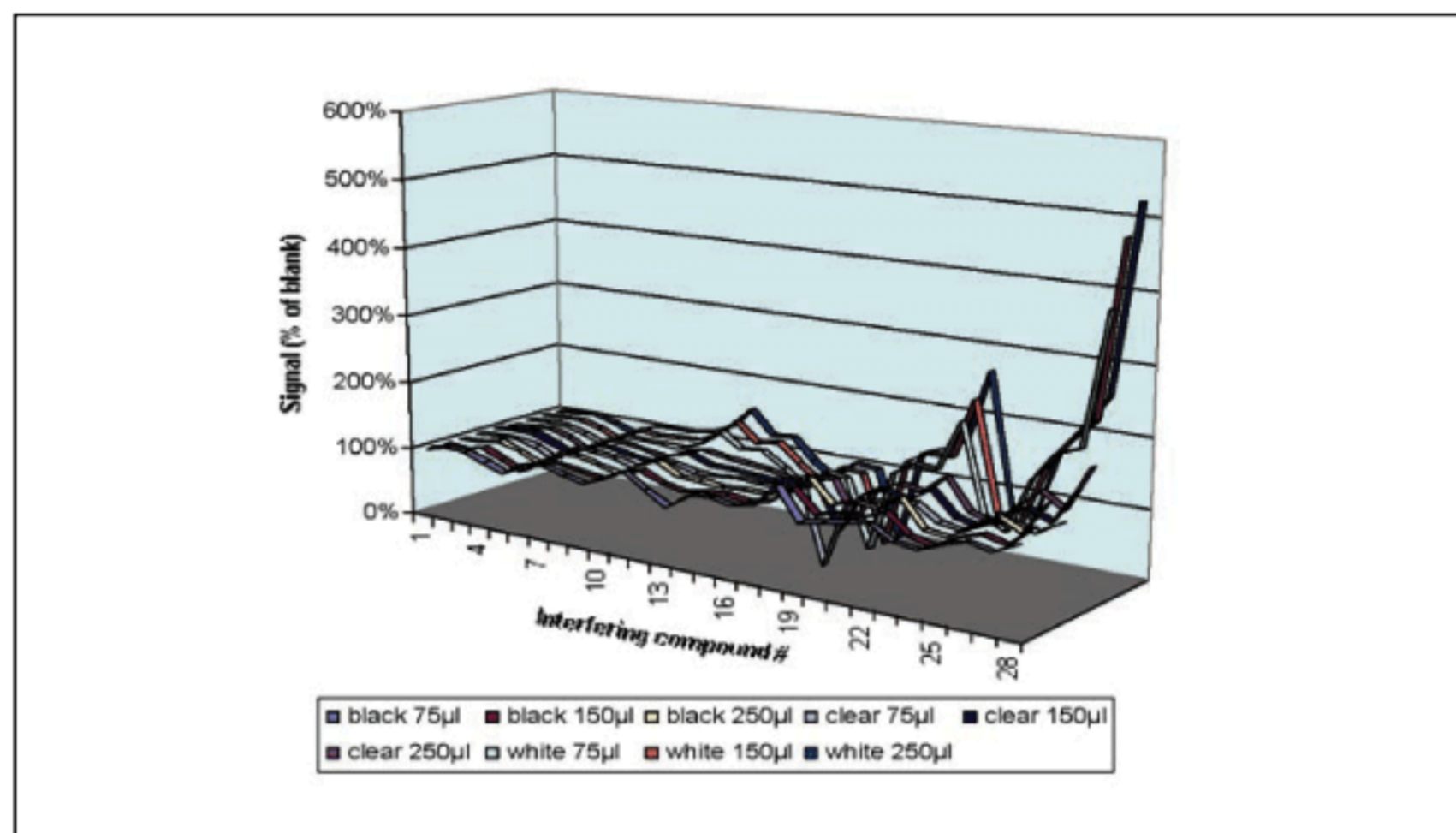


Figure 2. Effects of plate color and volume on the efficiency of quench correction. Interfering compounds are numbered according to the Table 1. The blank-corrected normalized ratio values (R_n) were compared to the values of a non-colored blank to compensate for different signal levels on black, clear and white plates. The assay was performed using two replicas.

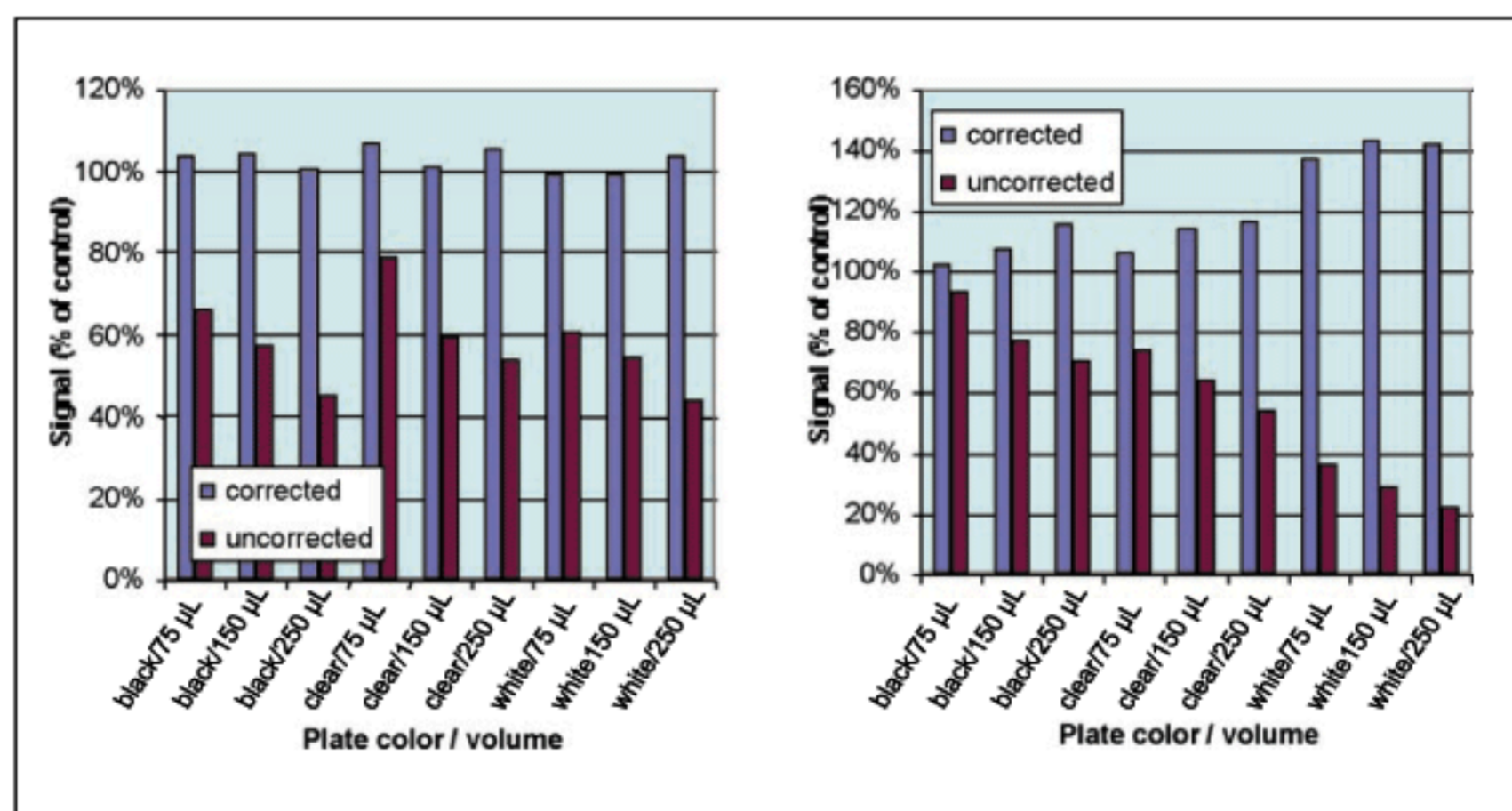


Figure 3. Comparison of corrected and uncorrected signals with different plates and volumes. In each plate the corrected and uncorrected values were compared to the sample without interfering substances. The colors used during these tests were 200µM tartrazine (A) and 10µM chicao sky blue (B).

CONCLUSIONS

According to Figures 2 and 3 there are differences between different colors in the efficiency of quench correction. Especially quenching caused by colors absorbing at the emission wavelength of europium cannot be effectively corrected without measurement of D absorbance (e.g. bromophenol blue in Figure 2. For more information see Hemmilä et. al. 1998). With almost all the tested colors the quench-correction equation worked most effectively when black plates and small volumes were used. Black plates have also been found to give the highest signal-to-noise ratios in kinase assays measured with VICTOR² (for more information see The Application Note "Miniaturization of LANCE™ Kinase Assays").

It should be noted that this equation is suitable only for time-resolved energy-transfer type assays such as the kinase assay. It cannot be applied to fluorescence quenching type assays, e.g. LANCE caspase (Karvinen et al. 1999) and helicase assays (Hurskainen et al. 1997).

REFERENCES

- Hemmilä, I. and Ahola, T. (1997) Homogeneous time-resolved fluorometric energy transfer assay (LANCE) for protein tyrosine kinase. Abstract of Papers Presented at the 3rd Annual Conference of the Society for Biomolecular Screening, Sept 1997, California
- Hemmilä, I., Oikari, T., Keränen, Y. and Blomberg, K. (1998) Multivariate analysis of interference in homogeneous screening assays. Paper presented at the 4th Annual Conference and Exhibition of the Society for Biomolecular Screening, Sept 1998, Baltimore MD, abs SAI-8
- Hurskainen, P., Virtanen, J., Liitti, P., Blomberg, K. and Hemmilä, I. (1997) Suitable substrates for homogeneous enzymatic DNA strand separation assays. Abstract of Papers Presented at the 3rd Annual Conference of the Society for Biomolecular Screening, Sept 1997, California
- Karvinen, J., Hurskainen, P., Liitti, P., Blomberg, K. and Hemmilä, I. (1999) Homogeneous time-resolved fluorescence quenching assay (LANCE™) for caspase-3. Poster presented at the 5th Annual Conference and Exhibition of the Society for Biomolecular Screening, Sept 1999, Edinburgh, Scotland, UK.

Selected LANCE products for kinase assay

CR130-100	SureLight™ Allophycocyanin-streptavidin (APC-SA), 1 mg
AD0066	LANCE Eu-W1024 labelled anti-pTyr antibody (PY20), 50 µg
AD0068	LANCE Eu-W1024 labelled anti-pTyr antibody (PT66), 50 µg
AD0161	LANCE Eu-W1024 labelled anti-pTyr antibody (PY100), 50 µg
AD0094	LANCE Eu-W1024 labelled anti-phosphothreonine antibody, 10 µg
AD0099	LANCE Eu-W1024 labelled anti-phosphothreonine-proline antibody (pTP101), 10 µg



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