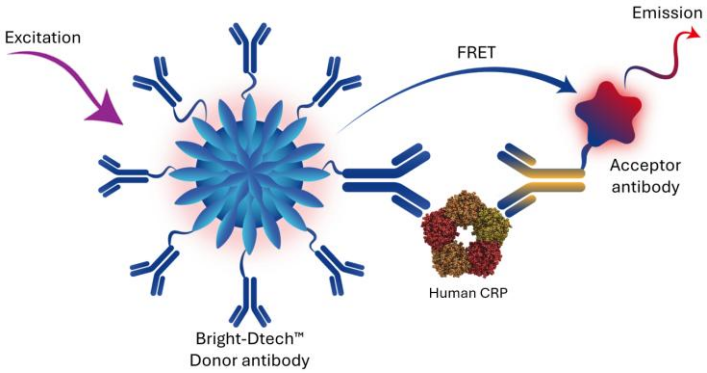
NoW-Dtech™
Human CRP Assay Kit
Ref: FREThCRP

1 | PRESENTATION

ASSAY KIT DESCRIPTION

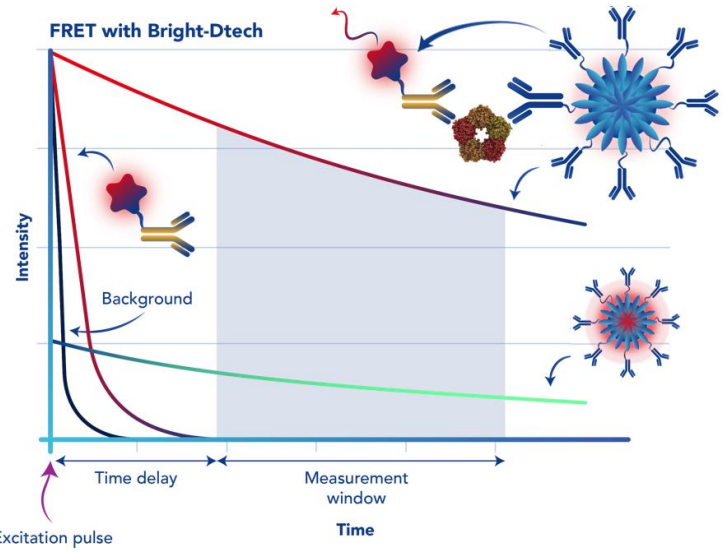


The Human CRP Assay Kit allows the quantitative detection of human CRP in serum using TR-FRET methodology. This kit incorporates Bright-Dtech™ technology, which enhances detection through its exceptional specificity and sensitivity. The assay range is 0.6-100 ng/mL.

TR-FRET ASSAY PRINCIPLE

Time-Resolved Förster Resonance Energy Transfer (TR-FRET) is a cutting-edge immunoassay technique widely used for the detection and quantification of biomolecules, including human CRP in serum. This method is fast, wash-free, reduces background fluorescence, and is cost-effective. Our Bright-Dtech™ technology offers a more sensitive (high brightness, leading to improved limits of detection) and stable (high photo-stability over days or weeks, resistance to photo-bleaching, and sustained signal intensity) approach.

In the NoW-Dtech™ TR-FRET method, two antibodies are employed to specifically detect human CRP. The first antibody is conjugated to our fluorescent nanoparticles (donor) and the second is labeled with fluorescent molecules (acceptor). Upon recognition of the target by both antibodies, energy is transferred from the donor to the acceptor, generating a detectable signal. This signal is measured using a time-resolved fluorescence reader, delivering highly specific and quantitative results.



2 | MATERIALS

KIT COMPONENTS

Human CRP (Standard stock)

Bright-Dtech™ Donor antibody

Acceptor antibody

TRF Buffer

384-well black microplate

MATERIALS REQUIRED BUT NOT SUPPLIED

- Vials.
- Precision single and multichannel pipettes with disposable tips.
- Adhesive sealing film.
- Plate reader with TR-FRET option.

3 | REAGENTS AND SAMPLE PREPARATION

STORAGE AND PRECAUTIONS

- The kit must be stored at 2-8°C and used before the expiration date indicated on the box.
- The instructions described below are for testing all the assay points of the kit. Adjust the volume according to the test desired.
- Do not substitute or mix reagents with those from other lots or other source.
- We can not guarantee the performance of the product outside the condition described.
- Bring all reagents, plate, standard and samples to room temperature prior to use and homogenize them with a vortex.

Important: Prepare the working standard dilutions and the samples first (see pages 3 and 4). Distribute them into the wells, then prepare the donor/acceptor mix solution (see page 3).

PREPARATION OF DONOR/ACCEPTOR MIX SOLUTION

Note: *The example bellow is for 384 points. For a different number of points, adjust the volumes according to Table 1. Follow the order of addition exactly.*

1. Sonicate (or vigorously vortex) the coupled nanoparticles **'Bright-Dtech™ Donor antibody'** to ensure a homogeneous solution.
2. Add 20 µL of **'Bright-Dtech™ Donor antibody'** stock solution to a vial containing 1976 µL of **'TRF Buffer'** and vortex.
3. Allow to stand for 5 minutes before adding the acceptor antibody.
4. Add 3.9 µL of **'Acceptor antibody'** to this solution and vortex again.

Points	96	192	288	384
Bright-Dtech™ Donor antibody	5.5 µL	11 µL	15 µL	20 µL
TRF Buffer	543 µL	1087 µL	1482 µL	1976 µL
Acceptor antibody	1.1 µL	2.1 µL	2.9 µL	3.9 µL

Table 1: *Preparation of Donor/Acceptor mix solution*

PREPARATION OF WORKING STANDARD DILUTIONS

1. Spin down the **'Standard stock'** tube briefly before use. Prepare a 1/100 pre-dilution using **'TRF Buffer'** as the diluent.
2. Use this 1/100 pre-dilution to prepare the first standard point at 100 ng/mL, using **'TRF Buffer'** as the diluent. Perform a 2-fold serial dilution to generate an 11-point standard curve, as shown in Figure 1. The following will yield sufficient volumes to run the standard dilution series in duplicate.
3. Diluted standards should be used immediately and should not stored for future use.

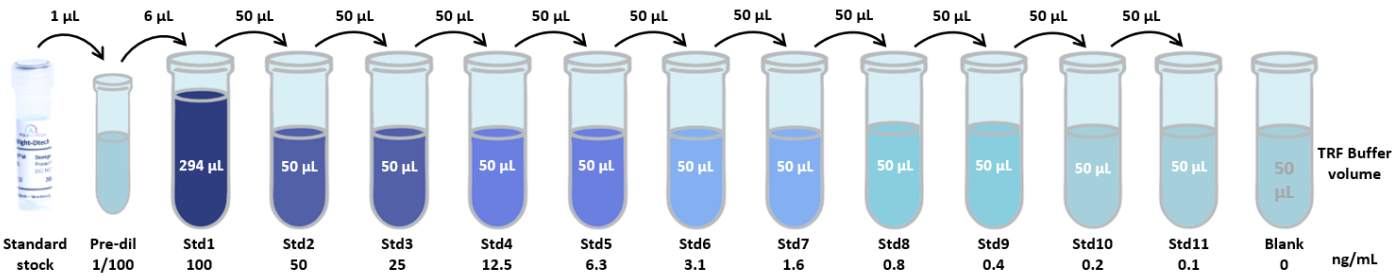


Figure 1: *Preparation of serial dilutions for the standard curve*

SAMPLE PREPARATION

- For serum sample dilutions: use TRF Buffer.
- Suggested dilution for normal serum : 7 000-fold or more.

Please note that levels of the target protein may vary between different samples. The optimal dilution factor for each sample must be determined by the investigator.

4 | ASSAY PROTOCOL

PROTOCOL OVERVIEW

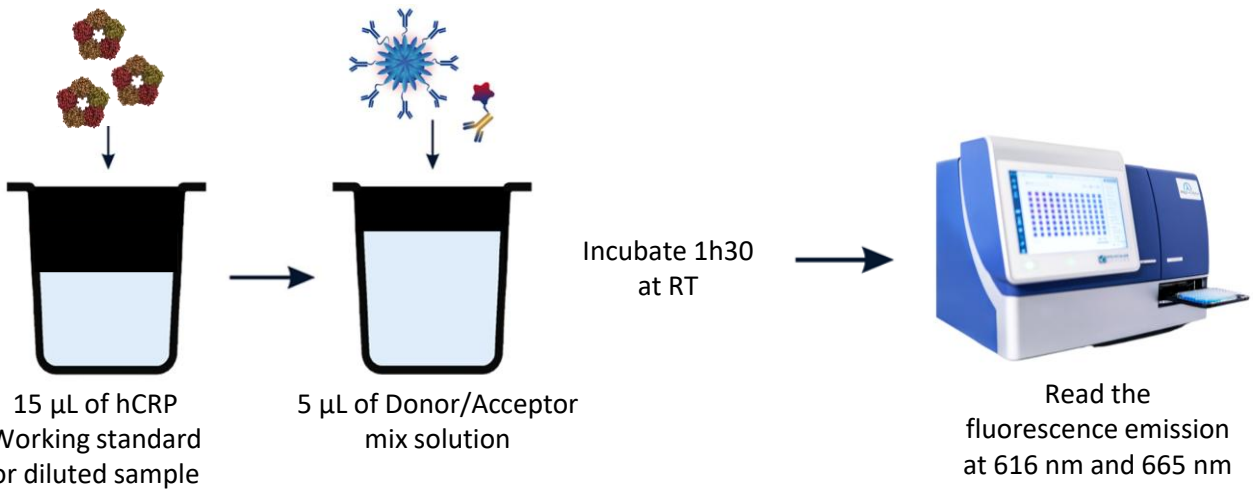
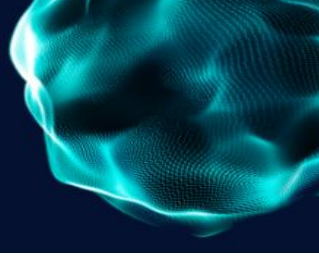


Figure 2: *Assay procedure summary*

1. Add 15 µL of each working standard or 15 µL of diluted sample to the appropriate wells.
2. Add 5 µL of the Donor/Acceptor mix solution to each well.
3. Cover the plate with adhesive foil.
4. Incubate for **1h30** at room temperature (RT).
5. Remove the adhesive foil.
6. Read the fluorescence at **616 nm** and **665 nm** using a TR-FRET-compatible microplate reader (excitation at **340 nm**).
7. Refer to '*TR-FRET plate reader settings*' section for detailed instructions.



TRF PLATE READER SETTINGS

- The instrument settings bellow are provided as a guideline only.
- Settings must be optimized for each reader (*).

Parameter	Setting
Excitation filter	340 nm
Emission filter	616 nm / 665 nm
Number of flashes *	110
Delay time *	30 µs
Integration time *	400 µs
Well scan	Multi-points

Table 2: *Example of instrument settings for a TRF reader*

5 | ANALYSIS

CALCULATIONS

- TR-FRET data are calculated ratiometrically using the following formula :

$$\text{TR-FRET Ratio} = \frac{\boldsymbol{Emission\ at\ 665\ nm}}{\boldsymbol{Emission\ at\ 616\ nm}} \text{ x } 10^4$$

- Create a standard curve by plotting the TR-FRET Ratio for each set of duplicate or triplicate standards on the Y-axis versus the corresponding human CRP concentration on the X-axis.
- The best-fit standard curve can be determined using nonlinear regression with a four- or five-parameter logistic (4PL or 5PL) curve-fit (sigmoidal dose-response curve with variable slope). We recommend using a commercial software program for this analysis.
- To determine the unknown human CRP concentrations in samples, locate the unknown TR-FRET Ratio values, insert them into the standard curve equation, and calculate the expected concentration. If samples have been diluted, the concentration obtained from the standard curve must be multiplied by the dilution factor.
- The intensity of the resulting signal is directly proportional to the concentration of the antigen present in the sample.
- Any undiluted or diluted sample reading greater than (or lower than) the highest (or lowest) standard should be further diluted (or less diluted) appropriately with 'TRF Buffer' and retested.

TYPICAL STANDARD CURVE

The standard curve below is an example only and should not be used to calculate results for unknown samples.

Results may vary between different TRF readers.

A new standard curve must be generated with each assay.

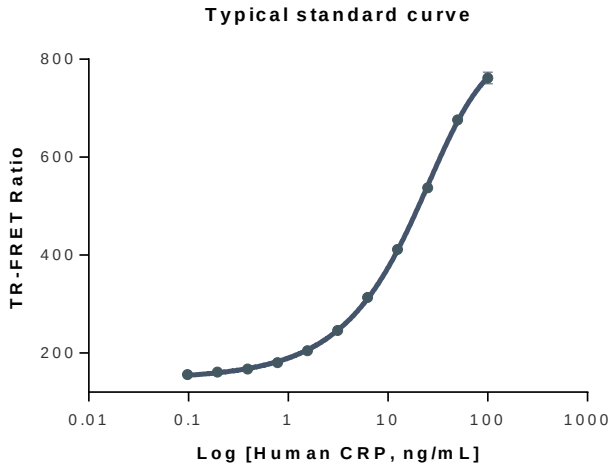


Figure 3: *A representative standard curve*

6 | PERFORMANCE CHARACTERISTICS

VALIDATION

Assay Type	Homogeneous sandwich immunoassay with Bright-Dtech™
Format	384-well plate
Hands-on time	20-30 minutes
Incubation time	1h30
Sample type (volume)	Diluted serum (40 µL for a duplicate)
Specificity	Human CRP
Limit of Detection (LOD)	0.11 ng/mL
EC_{50}	24 ng/mL
Dynamic range	0.6 – 100 ng/mL

Table 3: *Validation parameters resume*

SENSITIVITY

The Limit of Detection (LOD) is **0.11 ng/mL**. This was determined by adding three standard deviations to the mean value of 70 blank (zero) standard replicates, run across three independent assays, and calculating the corresponding concentration.

PRECISION

Intra-assay precision

Intra-assay precision was evaluated by measuring three concentrations (high, medium, and low) of human CRP spiked in TRF Buffer, with 20 replicate determinations. The intra-assay percentage coefficient of variation (%CV) was < 5%.

Spike	Number of replicates	%CV
High	20	4
Medium	20	4
Low	20	5

Table 4: *Intra-assay precision performance*

Inter-assay precision

Inter-assay precision was evaluated by measuring three concentrations (high, medium, and low) of human CRP spiked in TRF Buffer, with 20 replicates determinations in three independent assays. The inter-assay percentage coefficient of variation (%CV) was < 4%.

Spike	Number of replicates	%CV
High	3 x 20	4
Medium	3 x 20	1
Low	3 x 20	2

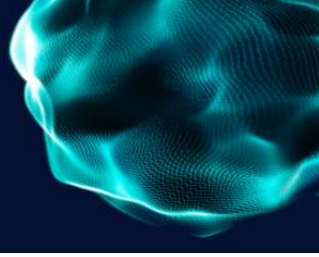
Table 5: *Inter-assay precision performance*

RECOVERY

To determine whether human CRP detection is affected by differences in the standard curve diluent and biological sample matrix, three different concentrations (high, medium, and low) of Human CRP were spiked into human serum depleted of CRP, diluted 1/7 000.

Spike	% Recovery
High	109
Medium	102
Low	102

Table 6: *Recovery performance*



LINEARITY

To evaluate the linearity of the assay, a high concentration of human CRP was spiked into human serum depleted of CRP, diluted 1/7 000. The sample was then serially diluted 2-fold up to 1/64 using TRF Buffer.

Dilution factor	% Linearity
1/2	103
1/4	94
1/8	99
1/16	102
1/32	97
1/64	103

Table 7: *Linearity performance*

7 | TROUBLESHOOTING GUIDE

Problem	Possible cause	Solution
Low or poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standards dilution	Prior to opening, briefly spin the standard stock tube. Check calculations, make new standard curve.
	Incorrect procedure	Check protocol (ensure reagents were added in the proper order).
No or poor signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation.
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation (review protocol).
	Incorrect procedure	Check protocol (ensure reagents were added in the proper order).
	Incorrect reader settings	Check filters/reader settings (ensure plate reader is set accurately for type of detection being used).
Large CV	Bright-Dtech™ Donor antibody not mixed well enough	Mix vigorously or sonicate the Bright-Dtech™ Donor antibody tube.
Low sensitivity	Improper storage of the kit	All the reagents should be stored according to the instructions.