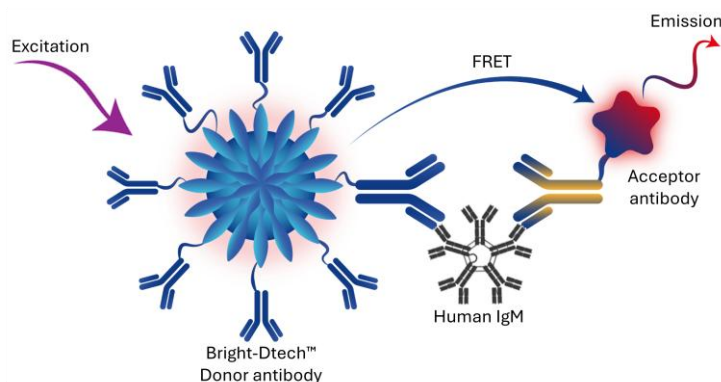


1 | PRESENTATION

ASSAY KIT DESCRIPTION

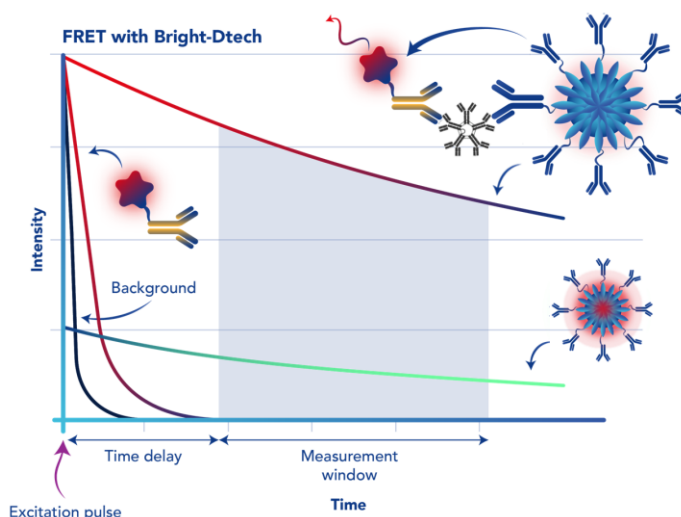


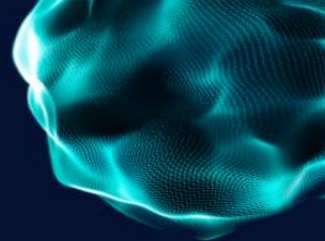
The Human IgM Assay Kit enables the quantitative detection of human IgM in serum using TR-FRET methodology. This kit incorporates Bright-Dtech™ technology, which enhances detection through its exceptional specificity and sensitivity. The assay range is 0.98-500 ng/mL.

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 IgM 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 IgM. 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, yielding highly specific and quantitative results.





2 | MATERIALS

KIT COMPONENTS



Human IgM-20X
(Standard stock)



Bright-Dtech™
Donor antibody



Acceptor
antibody (10X)



TRF Buffer



384-well black
microplate

MATERIALS REQUIRED BUT NOT SUPPLIED

- Vials.
- Precision single and multichannel pipettes with disposable tips.
- One adhesive sealing film is included, but others will be necessary.
- Plate reader with TR-FRET option.

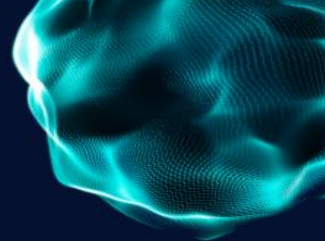
3 | REAGENTS AND SAMPLE PREPARATION

STORAGE AND PRECAUTIONS

- The kit must be stored at 2-8°C and used before the expiration date indicated on the box.
- Do not substitute or mix reagents from different lots or sources.
- We cannot guarantee the performance of the product if used outside the conditions described.
- Briefly spin down the tubes prior to use to remove any liquid that may have settled on the cap during shipping.
- Bring all reagents, plate, standard, and samples to room temperature prior to use, and homogenize them by vortexing. For the '**Bright-Dtech™ Donor antibody**' tube, sonication is strongly recommended instead of vortexing.



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



PREPARATION OF WORKING STANDARD DILUTIONS

1. Spin down the '**Standard stock (20X)**' tube briefly before use.
2. Prepare a 1X solution of '**Standard stock**' using '**TRF Buffer**' as the diluent. Add 1 μL of '**Standard stock (20X)**' to a vial containing 19 μL of '**TRF Buffer**'.
3. Use this 1X solution to prepare the first standard point at 500 ng/mL, using '**TRF Buffer**' as the diluent. Perform a 2-fold serial dilution to generate a 10-point standard curve, as shown in Figure 1. This procedure will yield sufficient volume to run the standard dilution series in triplicate.
4. For Blank points, perform 6 to 12 replicates using '**TRF Buffer**' only.

Note: The diluted standards should be used immediately and must not be stored for future use.

Standard	Serial dilutions	Working concentration (ng/mL)
Std 1X	1 μL Std stock (20X) + 19 μL TRF Buffer	-
Std 1	1 μL Std 1X + 109 μL TRF Buffer	500
Std 2	50 μL Std 1 + 50 μL TRF Buffer	250
Std 3	50 μL Std 2 + 50 μL TRF Buffer	125
Std 4	50 μL Std 3 + 50 μL TRF Buffer	62.5
Std 5	50 μL Std 4 + 50 μL TRF Buffer	31.25
Std 6	50 μL Std 5 + 50 μL TRF Buffer	15.6
Std 7	50 μL Std 6 + 50 μL TRF Buffer	7.8
Std 8	50 μL Std 7 + 50 μL TRF Buffer	3.9
Std 9	50 μL Std 8 + 50 μL TRF Buffer	1.9
Std 10	50 μL Std 9 + 50 μL TRF Buffer	0.98
Blank	150 μL TRF Buffer	0

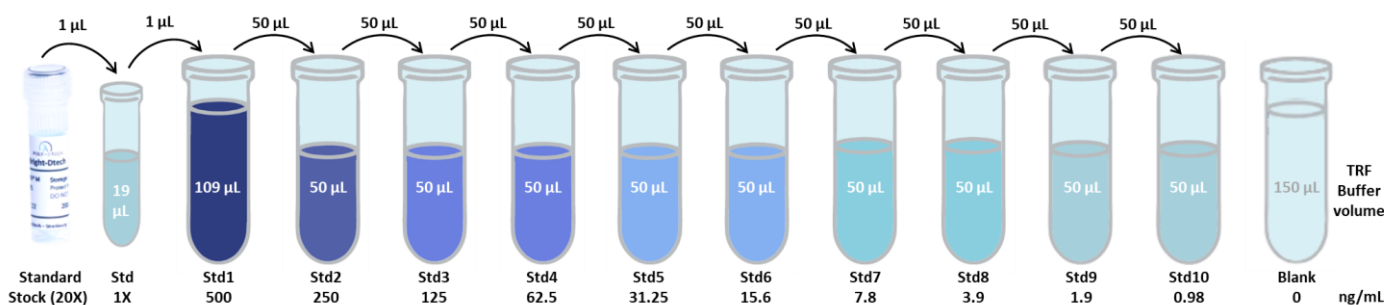
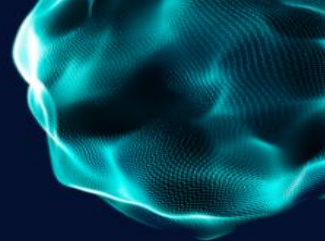


Figure 1: Preparation of serial dilutions for the standard curve



SAMPLE PREPARATION

- For serum sample dilutions: use TRF Buffer.
- Suggested dilution for normal serum : 10 000-fold or more.
- Prepare enough volume to add 10 µL per well (e.g., 40 µL if using duplicates).
- Take care to gently agitate patient samples to ensure homogeneity.

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.

PREPARATION OF DONOR / ACCEPTOR MIX SOLUTION

The example below is for 384 points (an entire plate). For a different number of points, adjust the volumes as indicated in Table 1.

 **Important:** Follow the order of addition exactly.

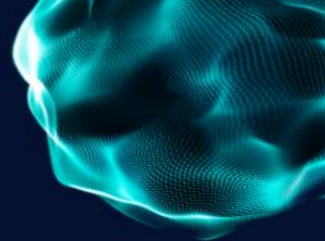
1. Sonicate or vigorously vortex (sonication is strongly recommended instead of vortexing) the **‘Bright-Dtech™ Donor antibody’** tube to ensure a homogeneous solution.
2. Add 25 µL of **‘Bright-Dtech™ Donor antibody’** stock solution to a vial containing 4 933 µL of **‘TRF Buffer’**, and vortex thoroughly.
3. Let the solution stand at room temperature for 5 minutes before proceeding to the next step.
4. Prepare a 1X solution of **‘Acceptor antibody’** using **‘TRF Buffer’** as the diluent. Add 5 µL of **‘Acceptor antibody (10X)’** to a vial containing 45 µL of **‘TRF Buffer’**.
5. Add 42 µL of the **‘1X Acceptor antibody’** to the solution prepared in step 2 and vortex again.

The donor/acceptor mix solution is ready to be distributed into the wells.

Note: The 1X Acceptor antibody solution should be used immediately and must not be stored for future use.

Points	96	192	384
TRF Buffer	1 381 µL	2 368 µL	4 933 µL
Bright-Dtech™ Donor antibody	7 µL	12 µL	25 µL
1X Acceptor antibody	12 µL	20 µL	42 µL

Table 1: Preparation of Donor/Acceptor mix solution



4 | ASSAY PROTOCOL

PROTOCOL OVERVIEW

1. Add 10 μ L of each working standard or 10 μ L of diluted sample to the appropriate wells.
2. Add 10 μ L of the Donor/Acceptor mix solution to each well.
3. Cover the plate with adhesive foil.
4. Incubate for **1.5 h** at room temperature (RT) if available on a microplate shaker set at 350 rpm.
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.

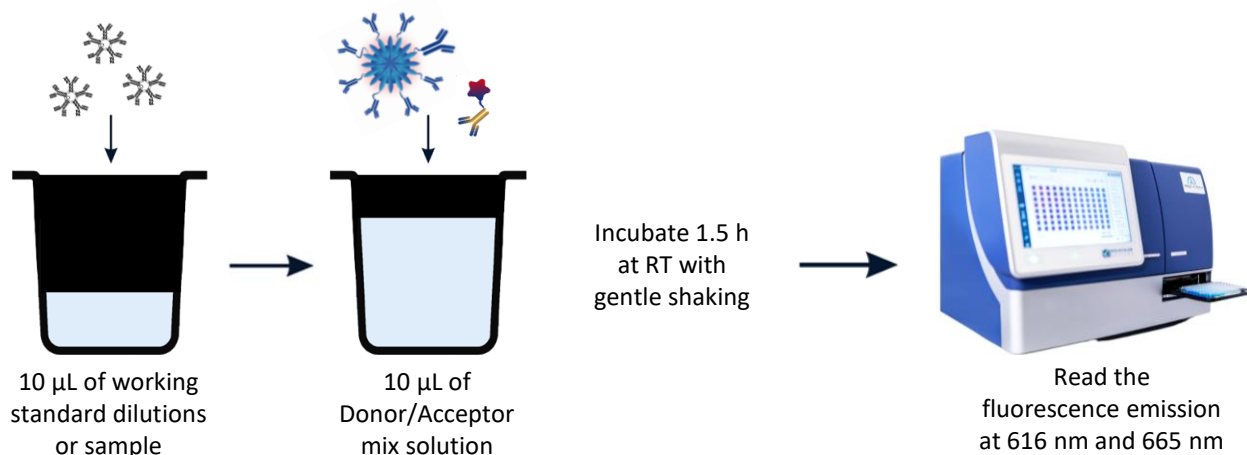


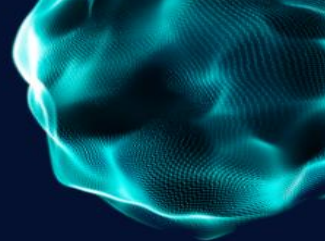
Figure 2: Assay procedure summary

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 μ s
Integration time *	400 μ s
Well scan	Multi-points

Table 2: Example of instrument settings for a TRF reader



5 | ANALYSIS

CALCULATIONS

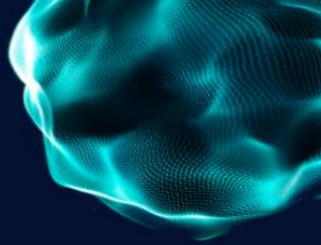
- Calculate the TR-FRET Ratio for each well using the following formula :

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

- Calculate the coefficient of variation (CV%) for each triplicate to assess the precision and consistency of the assay results using the following formula. The smaller the percentage, the more precise and consistent the data.

$$\text{CV\%} = \frac{\text{Standard deviation of the triplicate}}{\text{TR-FRET mean ratio of the triplicate}} \times 100$$

- Create a standard curve by plotting the TR-FRET Ratio for each set of triplicate standards on the Y-axis against the corresponding human IgM 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 IgM concentrations in samples, locate the TR-FRET Ratio values, insert them into the standard curve equation, and calculate the expected concentration. If the 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 sample reading greater than the highest standard should be diluted appropriately with 'TRF Buffer' and retested.
- Any diluted sample reading greater than the highest standard should be further diluted appropriately with 'TRF Buffer' and retested.
- Any diluted sample reading lower than the lowest standard should be less diluted appropriately with 'TRF Buffer' and retested.
- A high dose hook effect may occur in samples with very high human IgM concentrations. In this case, dilute the samples further to avoid this effect.



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.

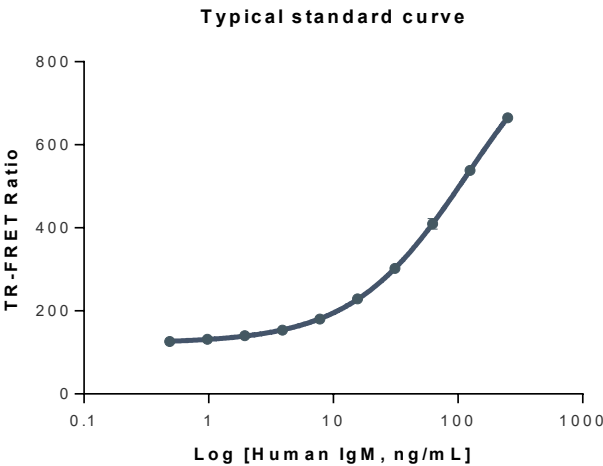


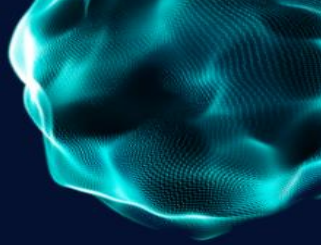
Figure 3: A representative standard curve

6 | PERFORMANCE CHARACTERISTICS

VALIDATION

Assay Type	Homogeneous sandwich immunoassay with Bright-Dtech™
Format	384-well plate
Hands-on time	20-30 minutes
Incubation time	1.5 h
Sample type (volume)	Diluted serum (20 µL for a duplicate)
Specificity	Human IgM
Limit of Detection (LOD)	0.5 ng/mL
Limit of Quantification (LOQ)	3.6 ng/mL
EC ₅₀	103 ng/mL
Dynamic range	0.98 – 500 ng/mL

Table 3: Validation parameters resume



S E N S I T I V I T Y

The Limit of Detection (LOD) is **0.5 ng/mL**. This was determined by adding three standard deviations to the mean value of 80 blank (zero) standard replicates, run across three independent assays, and calculating the corresponding concentration.

P R E C I S I O N

Intra-assay precision

Intra-assay precision was evaluated by measuring three concentrations (high, medium, and low) of human IgM spiked in TRF Buffer, with 16 replicate determinations. The intra-assay percentage coefficient of variation (CV%) was < 9%.

Spike	Number of replicates	CV%
High	16	4
Medium	16	5
Low	16	9

Table 4: *Intra-assay precision performance*

Inter-assay precision

Inter-assay precision was evaluated by measuring three concentrations (high, medium, and low) of human IgM spiked in TRF Buffer, with 16 replicates determinations in three independent assays. The inter-assay percentage coefficient of variation (CV%) was < 6%.

Spike	Number of replicates	CV%
High	3 x 16	6
Medium	3 x 16	6
Low	3 x 16	3

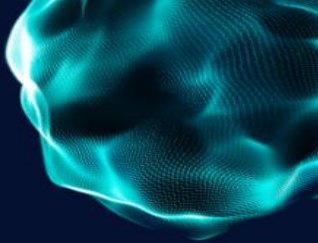
Table 5: *Inter-assay precision performance*

R E C O V E R Y

To determine whether human IgM detection is affected by differences in the standard curve diluent and biological sample matrix, three different concentrations (high, medium, and low) of Human IgM were spiked into human serum depleted of IgM, diluted 1/15 000.

Spike	% Recovery
High	99
Medium	87
Low	88

Table 6: *Recovery performance*



L I N E A R I T Y

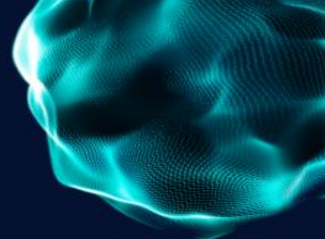
To evaluate the linearity of the assay, a high concentration of human IgM was spiked into human serum depleted of IgM, diluted 1/15 000. The sample was then serially diluted 2-fold up to 1/128 using TRF Buffer.

Dilution factor	% Linearity
1/2	107
1/4	93
1/8	102
1/16	105
1/32	104
1/64	97
1/128	95

Table 7: Linearity performance

7 | T R O U B L E S H O O T I N G G U I D E

Problem	Possible cause	Solution
Low or poor standard curve	Inaccurate pipetting.	Check pipettes.
	Improper standard dilution.	Before opening, briefly spin the standard stock tube. Check calculations and create a new standard curve.
	Incorrect procedure.	Check the protocol (ensure reagents were added in the correct order).
No or weak signal	Incubation times too short.	Ensure sufficient incubation times; switch to a 2.5 hours 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 correct order).
	Incorrect reader settings.	Check filters/reader settings (ensure the plate reader is set accurately for the type of detection being used).
High CV%	Bright-Dtech™ Donor antibody not mixed properly.	Sonicate or mix vigorously the Bright-Dtech™ Donor antibody tube.
Low sensitivity	Improper storage of the kit.	All reagents should be stored according to the instructions.



8 | PLATE LAYOUT

384 - well plate layout

- Example layout of a 384-well plate featuring a standard curve prepared in triplicate and 12 replicates for the blank. The remaining wells can be used for sample analysis or additional standard curves.

