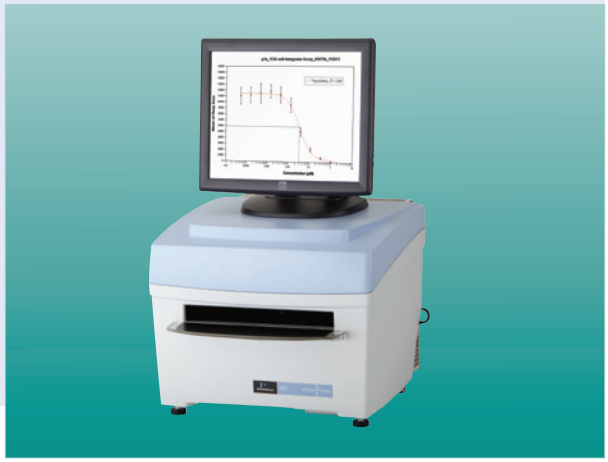
APPLICATION NOTE

Multimode Detection

Using the EnSpire Multimode Plate Reader to Measure IMAPlate-Based Rapid Miniature ELISA for the Quantification of Troponin I



Introduction

Reducing sample volumes is an effective method for conserving precious samples but conventional plate design has been a limiting factor. The IMAPlate™ 5RC96 overcomes these limitations by replacing conventional 96-well plates for carrying out reactions and assays. It comprises 96 identical, funnel-like reaction units which contain a 5 µL capillary reaction chamber with a light pathlength of 5 mm.

Besides being an economic, sensitive and flexible method of liquid handling, the unique self-dosed liquid take-up feature of the IMAPlate also supports manual high-throughput. In this case, up to 96 individual samples can be simultaneously loaded by simply dipping the open bottom of the capillary reaction chambers and then emptied by applying them to a filter paper.

Loaded plates can then be measured fluorimetrically or colorimetrically with a multimode plate reader such as the PerkinElmer EnSpire®. This plate reader is an excellent tool for measuring IMAPlate-based miniature assays, offering superior features over conventional assays such as dramatic reduction in sample and reagents consumption, and the time to results.

In this study we demonstrated that these advantages are of particular relevance for troponin I quantification assays. The cardiac troponin I (cTnI) plays an important role in the regulation of cardiac muscle contraction. It does not ordinarily exist in peripheral circulation but is released into circulation in myocardial necrosis. Therefore, the cTnI is a reliable cardiac biomarker for cardiac muscle injury. The amount of cTnI in peripheral circulation is usually quantified by enzyme-linked immunosorbent assay (ELISA), which is normally performed in conventional 96-microwell plates.

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Materials and Methods

Reagents and Materials

- Capture antibody (clone 560) and detection antibody coupled with HRP (clone MF4) , for cardiac troponin I, Hytest, Ref. 4T21
- Human cardiac troponin I-T-C complex, Hytest, Ref. 8T62
- TMB (Tetramethylbenzidine) substrate, Substrate Reagent Pack, R&D Systems, Ref. DY999
- IMAPlate™ 5RC96 starter kit
- Nunc 96-well Polysorp plates
- PerkinElmer plate reader: EnSpire 2300 Multimode Plate Reader
- Reagents: PBS pH 7.4; fat free powder milk; Tween-20, H_2SO_4
- Cardiac troponin I (cTnI) free serum, Hytest, Ref. 8TFS
- Washing buffer: PBS - 0.05% Tween 20
- Detection buffer: PBS - 0.05% Tween 20 - 0.2% fat free powder milk

The capture antibody and detection antibodies were diluted to 7.5 µg/mL in PBS and to 1 µg/mL in detection buffer, respectively.

IMAPlate protocol:

1. Coat the IMAPlate 5RC96 plate by directly pipetting 5 µL of capture antibody solution into the reaction chamber from the bottom opening. Incubate 30 minutes at room temperature in a high humidity environment to prevent from evaporation.

   [*When working with very low sample volumes, small interferences (e.g. bubbles, particles, homogeneity of solution) can have a large impact on the results. Make sure the sample solution is mixed thoroughly before pipetting. Reverse pipetting is recommended in order to avoid bubble formation or incomplete dispensing from the tip.*]

2. Wash the plate 4 times. Empty the IMAPlate by placing it onto a filter paper and pushing slightly against filter paper for 10 seconds to allow the solution to be completely absorbed. Touch-load the IMAPlate with wash buffer and empty the IMAPlate four times.

   [*Touch-load procedure: place the IMAPlate onto a plate cover containing sufficient wash buffer and move up and down several times to ensure the capillarity reaction chambers are fully loaded. Instead of the plate cover, a flat bottomed 96-well plate containing 100 µL of wash buffer in each well can also be used.*]

3. Block the IMAPlate by touch-loading with blocking solution. Incubate 10 minutes in a high humidity environment at room temperature.
4. Wash the plate 4 times (as in step 2).
5. Prepare cTnI standard concentrations at 12.5; 6.25; 3.125; 1.56; 0.78; 0.39; 0.2 and 0 ng/mL in PBS. Add standard or sample by directly pipetting 5 µL standard concentrations into the reaction chamber from the bottom opening. 2 or 3 replicates are prepared. Incubate for 30 minutes at room temperature in a high humidity environment.
6. Wash the plate 4 times (as in step 2).
7. Add detection antibody to the IMAPlate by directly pipetting 5 µL of antibody solution into the reaction chamber through the bottom opening. Incubate for 30 minutes at room temperature in a high humidity environment.
8. Wash the plate 4 times (as in step 2).
9. Add TMB substrate by touch-loading of TMB solution from a plate cover or a 96-well plate. Incubate for 5 - 20 minutes at room temperature.

   [*For a U- or V-bottomed 96-well plate, 25 µL TMB solution in the wells is enough and for a flat bottomed 96-well plate 80 – 100 is needed.*]

10. Pipette 0.5 µL of 3M H_2SO_4 stop solution directly into the reaction chamber from the bottom opening. Slowly invert the IMAPlate several times to make sure the solutions are mixed well. Alternatively, place the IMAPlate on a 96-well plate that contains the stop solution [*15 µL for a U- or V-bottomed 96-well plate and 80 µL for a flat bottomed 96-well plate*] and wait for several seconds to allow the solution in the reaction chamber to partially exchange with the stop solution through the bottom openings. Slowly lift the IMAPlate and invert several times to make sure the solutions are mixed well.
11. Measure absorbance at both peak wavelength (450 nm for TMB) and base-line wavelength (e.g. 650 nm for TMB) by using the IMAPlate adaptor according to the IMAPlate user manual. Calculate the true absorbance. ($Abs_{true} = Abs_{peak} - Abs_{baseline}$).

96-well plate protocol:

1. Coat the plate with 50 µL/well of capture antibody solution. Incubate overnight at 4 °C.
2. Wash the plate 4 times (200 µL/well wash buffer).
3. Block the plate with 100 µL of blocking solution to each well. Incubate for 2 hours at room temperature with agitation.
4. Wash the plate 4 times.
5. Prepare cTnI standards concentrations at 12.5; 6.25; 3.125; 1.56; 0.78; 0.39; 0.2 and 0 ng/mL in PBS. Add 50 µL/well standard or sample. Incubate for 2 hours at room temperature with agitation.
6. Wash the plate 4 times.
7. Add 50 µL/well of detection antibody solution. Incubate for 2 hours at room temperature with agitation.

8. Wash the plate 4 times.
9. Add 100 µL of TMB substrate to each well. Incubate for 5-20 minutes at room temperature with agitation.
10. Add 100 µL of stop solution to each well.
11. Measure absorbance at 450 nm according to TMB solutions instructions.

Results (from the evaluation report of Firalis®)

Standard curves:

Figure 1 shows the cTnI standard curves obtained from both the IMAPlate (red) and NUNC 96-well plate (blue). Two independent experiments were performed for the two different assays (IMAPlate and NUNC 96-well plate). Samples were run in triplicate for the IMAPlate assay and in duplicate for the NUNC 96-well plate assay. The standard curves from the IMAPlate and the 96-well plate are comparable. The maximum absorbance/background ratio is very close (41.31 fold background versus 42.31 for the IMAPlate and the 96-well Nunc plate, respectively) while the concentration of cTnI to reach half maximum absorbance/background is around 30% lower for the IMAPlate than the 96-well Nunc plate (4.2 vs 5.9 ng/mL for the IMAPlate and the 96-well Nunc plate respectively). Therefore, the data indicates that the IMAPlate has a better resolution towards lower concentrations.

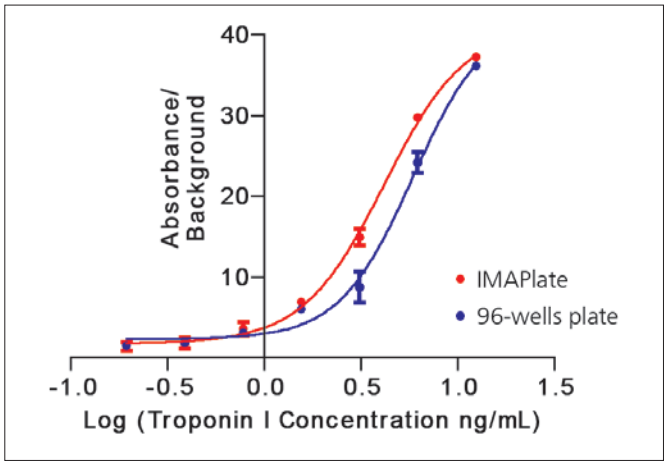

Figure 1. 4 PL fit of the absorbance/background ratio versus log of cTnI concentration using the Graphpad Prism® Software. Mean ±SD. N=1. The results are representative of the two independent experiments.

	IMAPlate	96-well plate
R^2	0.993	0.991
LOD	0.74 ng/mL	0.94 ng/mL

Table 1. LOD values (mean of 2 independent experiments for each assay, N=2).

Sensitivity, Limit of Detection (LOD):

Limit of detection (LOD) was calculated as: Average of background + 2.5 SD of background and are shown in Table 1. The IMAPlate offers a slightly better sensitivity compared to the conventional 96-well plate assay.

Intra-assay precision:

The CV% of the cTnI standards was usually less than 20% in triplicates for the IMAPlate assay and in duplicates for the 96-well plate assay, from cTnI concentrations from 1.56 to 12.5 ng/mL.

Recovery in serum

Additional samples have been prepared by spiking pure troponin–free serum with known concentrations of cTnI. No difference could be observed between the IMAPlate and 96-wells plate assays for concentrations of around 5 ng/mL.

Discussion

The recently developed IMAPlate technology is the world's first miniaturized analytical platform that also supports manual high-throughput liquid handling. Due to the very small dimensions of the capillary reaction chamber, use of high quality plate readers such as the EnSpire Multimode Plate Reader from PerkinElmer is recommended. The new rapid auto plate definition function makes the EnSpire Multimode Plate Reader much more attractive for measuring the IMAPlate and obtaining high quality data.

The miniature cTnI ELISA assay using the IMAPlate showed comparable results to a standard cTnI ELISA assay using conventional 96-well plates. The shape of the two standard curves was quite similar except that the results from the IMAPlate shifted towards lower concentrations. Combined with the lower limit of detection in the IMAPlate that was observed, this indicates a greater sensitivity of the IMAPlate-based cTnI ELISA. In addition, the requirement for samples and reagents was greatly decreased. The time-to-results is reduced from overnight coating plus 7 hours to less than 3 hours including the coating procedure. The unique self-dosed liquid take-up feature also leads to very simple washing steps. The touch loading procedure requires minimal practice.

In conclusion, the PerkinElmer EnSpire Multimode Plate Reader is ideally suited for the IMAPlate technology. The combination of these two technologies provides a very useful miniature lab tool for routinely performing assays and analyzing samples.

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