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A HOMOGENEOUS CASPASE 3 ACTIVITY ASSAY USING HTRF® TECHNOLOGY

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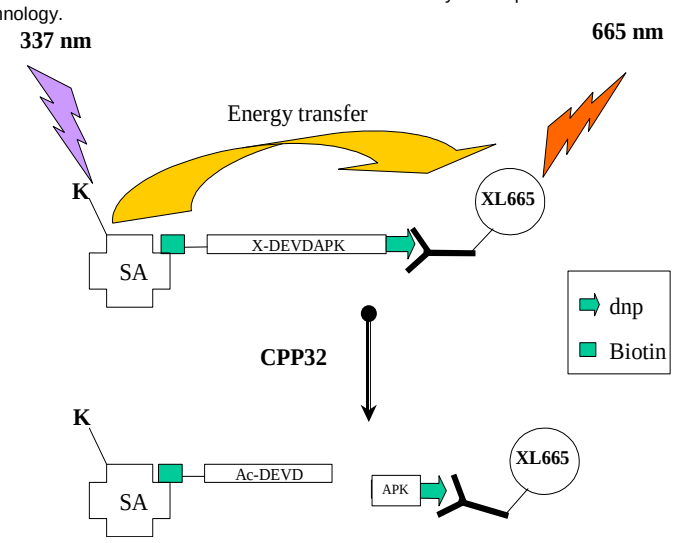
INTRODUCTION

Caspases (cysteinyl-aspartic-acid-proteases) are specific proteases, which play a key role in the mammalian apoptotic machinery. CPP32 belongs to the caspase 3 family and becomes activated during apoptosis. Its activation is responsible for the cleavage of several cellular components related to DNA repair and regulation (1). A CPP32 activity assay has been set up using HTRF® technique derived from TRACE® technology (2). The technology is based on non-radiative energy transfer from the europium cryptate donor to the cross-linked allophycocyanine (XL665) acceptor (3,4) leading to a long-lived fluorescent signal. The efficiency of the transfer depends on the distance between the acceptor and the donor. In this assay, a doubly labeled substrate (biotin and DNP (dinitrophenyl)) was used, allowing the use of two generic reagents.

This cassette format has thus allowed to increase the easiness of assay developments and to be amenable to HTS format using HTRF® technology.



MATERIALS AND METHODS

CPP32 with a C-terminal HIS_6 tag bound to Ni-NTA-agarose beads were purchased from Upstate Biotechnology Incorporation (NY USA Cat #14-212) Prior to implementing the enzyme assay, CPP32 enzyme was released from the beads by incubating with 5ml of 0,3 M imidazol containing 5mM DTT for 90 mn at 25°C.

- The CPP32 peptide substrate, biotin-X-DEVDAPK(dnp)-NH^2 for HTRF® assay was purchased from Peptide Institute (Osaka, Japan).
- The CPP32 inhibitor, Ac-DEVD-CHO was purchased from Bachem Incorporation (Switzerland Cat #H-2496).
- Natural product samples were provided by natural product chemistry group of Shionogi Discovery Research Laboratories in DMSO
- XL665-labeled anti-dnp antibody and crypate-labeled streptavidin were made at Cis Biointernational (Marcoule, France).
- Potassium fluoride was from Sigma Chemical (Cat # P-1179)

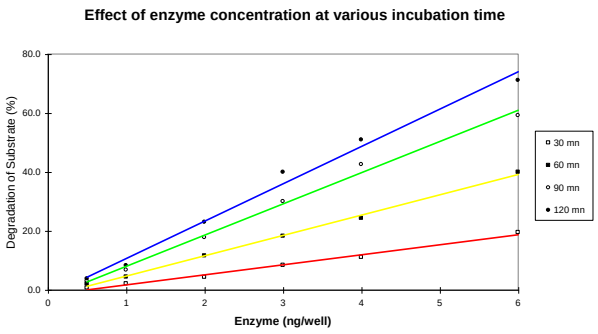
HTRF® format

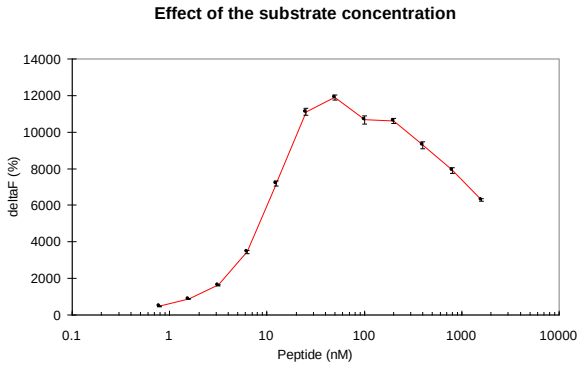
One microliter of sample or DMSO (as a control) was added into the Sumilon black 96-well microtiterplates. A volume of 34 µl of CPP32 (3 ng) in the reaction buffer (HEPES 100 mM pH 7.5, 1 mM EDTA, 5 mM DTT, 0 ;1% CHAPS, 10% glycerol) was then added to each well. Finally, the peptide substrate biotin-X-DEVDAPK(dnp)-NH_2 (22 nM final concentration) was added to each well for a total volume of 50 µl.
Plates were incubated for 90 mn at 25°C. After incubation, 150 µl of HTRF® reagents (1.3 nM Europium cryptate-labeled streptavidin and 76 nM XL665-labelled anti-dnp antibody in phosphate buffer 100 mM pH 7.4, 0,6 M KF, O,1% BSA) was added to detect the remaining substrate.
After one hour incubation at 25°C, the time resolved fluorescence was measured by Packard Discovery® reader (λ_{ex}: 337 nm, λ_{em}: 620 nm and 665 nm)

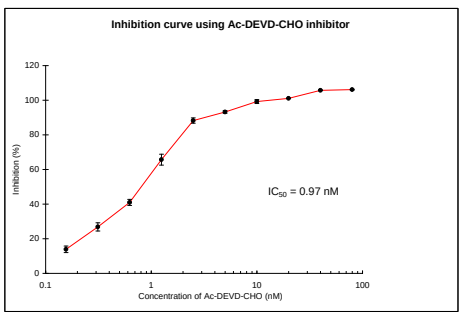
HTRF® plate reader and data handling

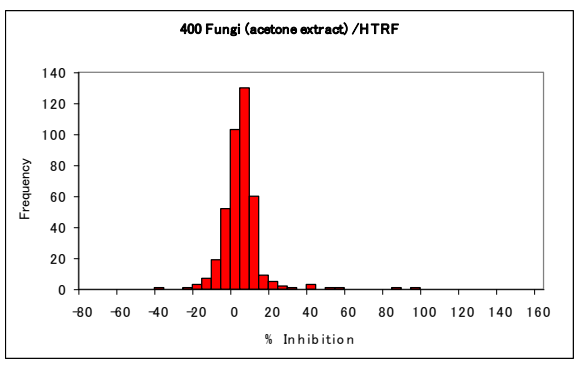
Plates were read on the Discovery® fluorometer with a 50 µs time delay and 400 µs read (average of 20 readings per second). Simultaneously, detection of fluorescence emission at 620 nm and 665 nm allowed ratioing and gave corrected results using Europium cryptate signal as an internal reference to correct for media absorbency by colored compounds in real time.
All results were expressed as Ratio (signal 665nm / signal 620 nm) or % of Delta F ($R_{665/620\ Pos} - R_{665/620\ Neg}$) / $R_{665/620\ Neg}$

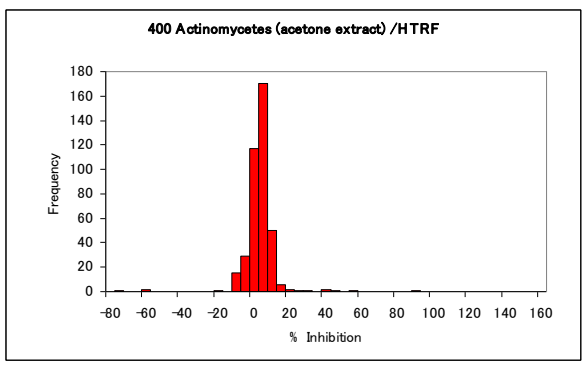
RESULTS

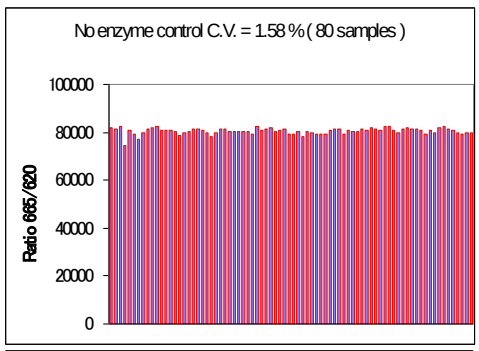


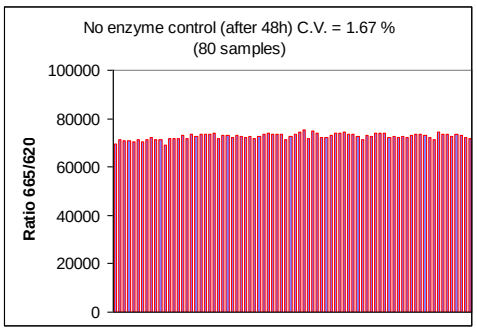


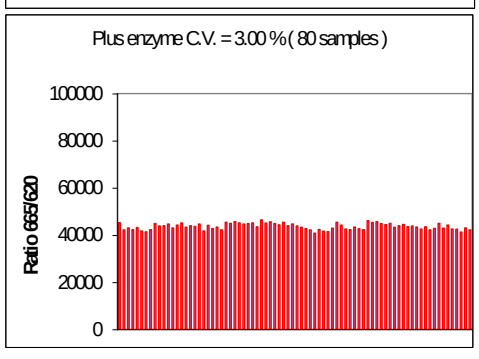


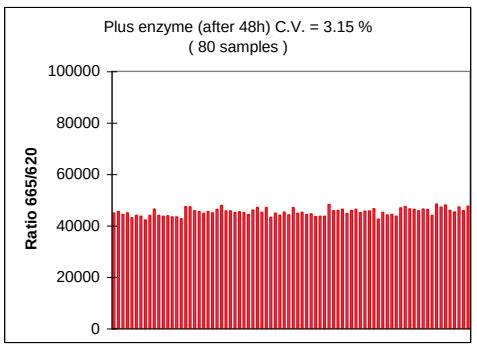












Assay development

Optimizing reagent concentrations for HTRF® assay required some constants. Baseline signal was defined by the emission at 620 nm, that is directly related to the Europium cryptate concentration. A minimum 620 nm signal was set at 50000 cps as recommended to allow a good correction of potential inner filter effect.
In this particular format, the maximum signal was obtained without enzyme and a decrease of the energy transfer was observed when protease activity occurred . The advantage of the "cassettes" format is the easiness of the adaptability for other similarly tagged targets.
To avoid possible reagents consumption, the determination of the minimum amount of enzyme and substrate per test was an important aspect of the assay optimization.
The data obtained with increasing enzyme concentration (from 0.5 ng/ well to 6 ng/ well) and increasing concentration of substrate (from 0.78 nM to 1600 nM) at various incubation time (from 30 mn to 120 mn) showed that 3 ng/ well of enzyme and 22 nM of substrate were a good compromise in term of reagents consumption, signal and linearity of the response.
To evaluate the HTRF® format, one important aspect was to demonstrate that the signal generated was caused by the simultaneous binding of XL665-labelled anti-DNP antibody and cryptate-labeled streptavidin on the peptide substrate. This was proved when signal was completely inhibited by increasing concentration of well known CPP32 inhibitor (Ac-DEVD-CHO).

Accuracy and stability of the HTRF® signal:

Peptide substrate was preincubate with enzyme(positive) or buffer (negative) for 90 mn at room temperature followed by incubation with HTRF® reagents for one hour and 48 hours prior to read on Packard Discovery® instrument. It is interesting to compare the HTRF® signal obtained with enzyme after one hour and 48 hours and without enzyme after one and 48 hours .In this experiment, 16 controls per plate (8 positives and 8 negatives) and ten plates were measured to offer good statistical evaluation.
The C.Vs. obtained were dramatically low and demonstrated the remarkable accuracy and stability of the system.

Robustness of HTRF® methodology:

One of the concerns with HTS assay is that it might give an unacceptably high hit rate. In an attempt to predict the hit rate, a small selection of natural product samples was tested under screening conditions. Results were obtained using Discovery® reader as delta F values which were converted into percentage inhibition and frequency distribution graphs were plotted for the 400 actinomycetes samples and the 400 fungi samples. The very interesting point is that HTRF® results reflected dramatically tight distribution throughout and presented a mean value clustered around 0% of inhibition. This experiment proved the robustness of the HTRF® methodology and its capability to easily discriminate potential genuine inhibitors.

CONCLUSION

CPP32 protease assay was chosen to evaluate HTRF® as a potential tool for High Throughput Screening. This assay presented some problems, complexity being based on 4 components, potential steric hindrance due to the small size of the substrate.

The use of « generic » reagents (Cryptate labeled streptavidin and XL665-labelled anti-DNP antibody) allows rapid assay development of similarly tagged substrates.

The tight distribution of negative samples allows an easy discrimination between positive and negative samples reducing therefore the hit rate which is of critical importance in HTS.

This behavior is in accordance with the assays already described in the literature (4) and seems to be a characteristic of the technology. Basically it may originate from the trully homogeneous format of the assay avoiding non specific interaction with solid phases. Ratioing the 665 specific signal by the 620 cryptate reference signal allows to alleviate inner filter effect variations which could be disastrous particularly with a natural product library.

The very low C.V. obtained under HTS conditions are also a characteristic of the technology. This is due partly to the suppression of separation / washing steps of the ELISA type assays but also by the ratio method.

Finally, HTRF® assay as yielded a low number hit substances (which are under investigation at this time) during HTS. The easiness (no separation), high reproducibility and accuracy ,high robustness, long time signal stability and high sensitivity demonstrate the interest of HTRF® as a new potential method for HTS assays.

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