A Fluorimetric Immunocapture Assay for Specific Detection of Active Matrix Metalloproteinase-2 in Biological Samples Using a 5-FAM/QXL® 520 FRET Peptide

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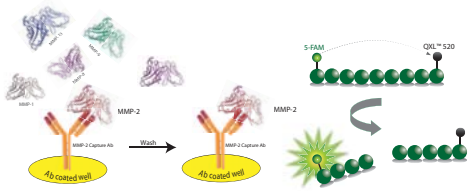
Introduction

Matrix metalloproteinase-2 (MMP-2), also known as gelatinase A, and Type IV collagenase, belongs to a family of MMPs. Natural substrates for MMP-2 are collagen, fibronectin, lamin, and elastin. Since MMP-2 plays a key role in angiogenesis, tumor cell invasion, and metastasis, this protease is considered one of the therapeutic targets for cancer. Different techniques have been developed for measurement of MMP-2. These are gelatin zymography, ELISA, and fluorescence resonance energy transfer (FRET) assay. However, these techniques have their shortcomings. Zymography is a semi-quantitative and relatively low-throughput method. ELISA measures only the amount of MMP-2, not the protease activity. FRET activity assay using a peptide substrate is a simple, fast, and sensitive for measuring MMP-2 activity. A major drawback is that its specificity is poor when using biological samples. In biological samples, multiple MMPs and other proteases present in these samples can generate non-specific cleavages. To solve this problem, we utilized an immunocapture technique in addition to the FRET assay. Monoclonal antibody to human MMP-2 was coated onto a 96-well plate and MMPs containing test samples were added to the microplate wells. This resulted in MMP-2 first being pulled down from the mixture by the pre-coated antibody. To detect enzyme activity, the MMP-2 peptide substrate with 5-FAM/QXL® 520 FRET pair was then added to the plate after removal of unbound proteins. Active MMP-2 cleaved the FRET substrate into two separate fragments resulting in release of 5-FAM fluorescence, which can be monitored. Fluorescence signal is correlated with MMP-2 enzyme activity. This assay can detect the MMP-2 activity in nanogram range and is ideal for specific defection of MMP-2 activity in biological samples containing multiple MMPs and other proteases.

Materials and Methods

- SensoLyte® Plus 520 MMP-2 Assay Kit
- √ MMP-2 5-FAM/QXL® 520 FRET substrate- designed and synthesized by Fmoc solid phase peptide synthesis method
- √ Monoclonal antibody to human MMP-2
- √ 4-aminophenylmercuric acetate (APMA) and assay buffer
- ARP101-selective inhibitor of MMP-2 & Batimastat-broad spectrum MMP inhibitor
- Human recombinant MMP-1, 2, 3, 7, 8, 9, 10, 12, 13, 14, 15 and 16
- CHO and U937 cell lines (ATCC, Manassas, VA)
- Preparation of cell culture supernatant containing MMPs: Plasmids encoding MMP-2, 8, and 14 (OriGene, Rockville, MD) were individually and transiently transfected to CHO cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY). Supernatant was collected at 24h after transfection and concentrated with an Amicon Ultra centrifugal filter device (Millipore, Billerica, MA, Cat# UFC905096). The concentrates were stored at -80°C.
- Immunocapture MMP activity assay: Samples containing MMPs were activated with 1 mM APMA at 37ºC for 1-2h before and after adding to the 12X8 black opaque strip plate pre-coated with the antibody. After 2h incubation at RT for immunocapture, the plate was washed and 5-FAM/QXL® 520 FRET peptide substrate was added and incubated for 1-24h at RT. Fluorescence was recorded by FlexStation 384II (Molecular Devices, Sunnyvale, CA) at Ex/Em=490/520nm.

Assay Principle



Scheme 1. MMP-2 in biological samples is captured by immobilized anti-MMP-2 antibody, and its proteolytic activity measured by 5-FAM/QXL® 520 FRET peptide substrate. The fluorescence signal is monitored at Ex/Em=490/520 nm.

Results

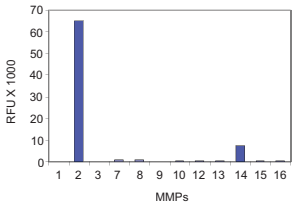


Figure 1. Specificity of immunocapture MMP-2 activity assay. APMA-activated recombinant MMP-1, 2, 3, 7, 8, 9, 10, 12, 13, 14, 15, and 16 at 30 ng each were added to a plate pre-coated with anti-MMP-2 antibody. After incubation, the plate was washed and activities of MMPs detected by a 5-FAM/QXL® 520 FRET peptide. Fluorescence (RFU) was monitored at 18h after adding the FRET peptide. Reading from all wells was subtracted with the reading from blank control, which contained FRET peptide but no MMPs (n=3, mean±S.D.).

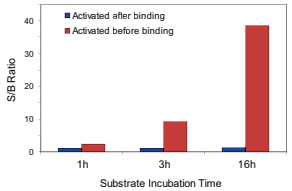


Figure 2. Effect of APMA activation on MMP-2 activity. Recombinant pro-MMP-2 was bound to pre-coated antibody first and then activated with 1 mM APMA for 1h at 37ºC (Blue bars). Recombinant pro-MMP-2 was activated first with 1 mM APMA for 1h at 37ºC and then bound to pre-coated antibody (Red bars). Activities of MMP-2 were measured by the cleavage of 5-FAM/QXL® 520 FRET peptide.

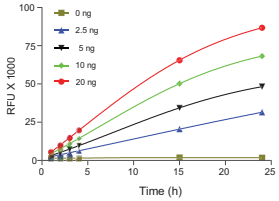


Figure 3. Time response of immunocapture MMP-2 activity assay. Different amount of APMA-activated MMP-2 (ng/well) was added to the anti-MMP-2 antibody pre-coated plate. MMP-2 activity was measured by the cleavage of 5-FAM/QXL® 520 FRET peptide. Fluorescence was recorded at different time after addition of the FRET peptide.

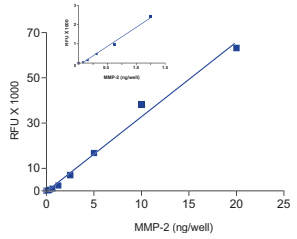


Figure 4. The sensitivity of immunocapture MMP-2 activity assay. APMA activated MMP-2 was serially diluted, and added to the anti-MMP-2 antibody coated plate. MMP-2 activity was measured by the cleavage of 5-FAM/QXL® 520 FRET peptide. Fluorescence (RFU) at 18h versus the amount of MMP-2 is plotted. The assay can detect as low as 1.0 ng/mL of active MMP-2 (n=3, mean±S.D.).

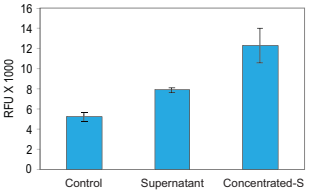


Figure 5. Detection of MMP-2 activity in U937 cell culture supernatant. U937 cells were seeded at a density of $1x10^6$ cells/mL in culture medium. After 24h, cell culture supernatant was collected and concentrated. Each APMA activated sample was added to the plate for immunocapture. MMP-2 activity was measured by the cleavage of the 5-FAM/QXL® 520 FRET peptide (n=3, mean±S.D.).

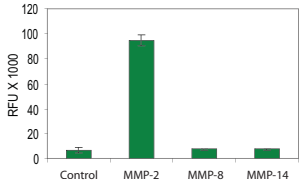


Figure 6. Detection of MMP-2 activity in culture supernatant of transfected CHO cells. CHO cells were seeded at a density of $2x10^5$ cells/mL on a culture plate. The next day, cells were transfected with MMP-2, -8, and -14 plasmids, individually. Cell culture supernatant was collected at 24h after transfection and concentrated. Each APMA activated sample was added to plate for immunocapture. MMP-2 activity was measured by the cleavage of the 5-FAM/QXL® 520 FRET peptide (n=3, mean±S.D.).

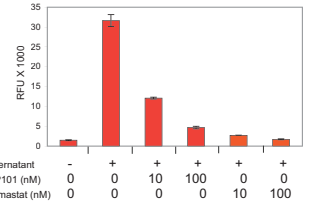


Figure 7. Inhibition of MMP-2 activity by ARP101 and Batimastat. CHO cells were transfected with MMP-2 plasmid and cell culture supernatant collected at 24h and concentrated. APMA activated sample was added to the plate for immunocapture. Plate was washed followed by the addition of inhibitors and 5-FAM/QXL® 520 FRET peptide. MMP-2 activity was measured by the cleavage of the FRET peptide (n=3, mean±S.D.).

Conclusions

- We utilized immunocapture and FRET techniques to develop the SensoLyte® Plus 520 MMP-2 Assay Kit. This new assay combines the specificity of ELISA with the convenience and sensitivity of 5-FAM/QXL® 520 FRET peptide substrate, thus enabling nanogram level detection of active MMP-2.

- The assay was evaluated for detection of MMP-2 activity with biological samples using U937 cells and transfected CHO cells, making the SensoLyte® Plus 520 MMP-2 Assay Kit ideal for specific detection of MMP-2 activity in biological samples which contain multiple MMPs and other proteases.

- The assay was validated with ARP101, a selective inhibitor of MMP-2, as well as with Batimastat, a broad spectrum inhibitor of MMPs.