SEKISUI XENOTECH

Product Instruction

IgG Catabolism Protocol
Lysosome / Tritosome Technical Tips

IgG catabolism reactions

Analysis of IgG catabolism by human lysosomes involves enzyme incubation, SDS PAGE, immunoblotting, ECL, and digital imaging. The data generated from use of this protocol is presented on the human lysosome flyer. Typical reaction volume ranged from 100-200µL depending upon the number of time points used in the experiment. The concentration of the IgG target used by Sekisui XenoTech was much lower than what is typically used in ADC catabolism studies (0.5-10 µM) for ease of analysis based on the linear range of detection of ECL signal by the digital imager.

High purity water	70 µL
10x catabolic buffer (K5200)	20 µL
Lysosomes (H0610.L diluted 1:10 in water = 0.25 mg/mL)	100 µL
IgG (diluted to 62 ng/µL, or approx. 475 nM)	10 µL
	200 µL total

Lysosomes were diluted just before use and only for the amount needed for the experiment. Lysosomes can undergo up to 8 freeze/thaw cycles without loss of cathepsin B activity; however the effect on other lysosomal enzymes is unknown.

Reactions were vortexed and incubated at 37°C in the capped tubes (0.6 mL, polyethylene).

At the appropriate times, 50 µL aliquots were removed from the incubation and heat inactivated at ≥95°C for 10 minutes. Snap freezing sample in liquid nitrogen or solubilizing sample in organic solvent can also be utilized to stop the reaction.

The remaining reaction volume was vortexed and continued to be incubated at 37°C.

Analysis of IgG catabolism was performed by standard immunoblot protocols with anti-Human IgG–HRP conjugated antibody and detected by enhanced chemiluminescent and digital imaging. Mass spectrometry, immunoblotting, ELISA, etc. are all appropriate analysis methods.

There are many uses for lysosomes/tritosomes, so experimental conditions should be optimized based upon your research goals. Sekisui XenoTech has examined catabolic activities using human lysosomes; however, it is believed that many of the conditions can also be utilized with rat tritosomes. Outlined below are a few things that need to be taken into account when optimizing the lysosome or tritosome matrix.

Technical Tips

Depending on the detection method of substrate catabolism, there are a few things that need to be taken into consideration:

1) Sekisui XenoTech defines cathepsin B activity units as: 1 unit will release 1 micromole of 7-amino-4-methylcoumarin from Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-RR-AMC) / minute at pH 5.5 at 38°C. Activity unit definitions need to be taken into account when comparing cathepsin B activity from different sources.

2) Rat tritosomes contain the surfactant Tyloxapol (nonionic polymer of the alkyl aryl polyether alcohol type). Loading lysosomes with Tyloxapol prior to isolation is essential for tritosome production and is unavoidable. It is unknown how much Tyloxapol is associated with the tritosomes post-isolation, it is up to the end users to determine if/how the presence of the surfactant may affect their research.

3) Endogenous intra- and extra-cellular lysosomal substrates are found in the lysosomal isolations (antibodies, growth factors, damaged cellular organelles, etc.). For example, there are endogenous IgGs present in the isolated lysosomal fractions as the IgG are constitutively undergoing endocytosis and degradation in the lysosomes. Thus, the lysosomal protein concentration will need to be adjusted/optimized for individual needs based on the limits of detection of the chosen analytic method and/or design of the experiment. The best way to control for this is to have a reaction that does not contain the biologic, but contains the lysosomal matrix.

4) Another way to address potential background due to endogenous peptides is to dilute the lysosomes/tritosomes to an acceptable limit, however this might dilute out the catabolic activity too much, so one has to find a balance. Sekisui XenoTech recommends a lysosomal protein concentration of 0.125 mg/mL as a starting point for experiment optimization. This concentration gives low endogenous IgG background with good catabolic activity (catabolizes >100 ng of exogenous IgG in 4 hours at 37°C) as assayed by immunoblotting. Immunoblot is very specific and sensitive; you may be able to dilute less, have higher catabolic activity, and still have acceptable background. The users will need to determine background/signal to noise ratio that will be appropriate for their research.

5) The environment inside the lysosome has been described both as reducing (Mego, 1984; Arunachalam et al., 2000; Maric et al., 2001; Pillay et al., 2002; Balce et al., 2014) and as oxidizing (Feener et al., 1990; Austin et al., 2005; Yang et al., 2006) in the literature with evidence for both; however, reducing agents are widely used in the reaction buffers of many *in vitro* catabolic enzyme reactions (typically 2.0-2.5 mM Dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine). Sekisui XenoTech has found that a reducing environment is necessary for full catabolic activity of the isolated lysosomes using purified human IgG or Z-RR-AMC as substrates. Sekisui XenoTech offers a convenient 10x catabolic buffer (K5200) that optimizes catabolic activity, however the buffer contains DTT and it may have an effect on target stability and structure, especially if structure is dependent upon disulfide bonds. If the presence of a reducing compound is unwanted, the DTT can be conveniently excluded from the reaction buffer prior to thawing K5200 for the first time by simply inverting the tube and removing the frozen DTT pellet prior to thawing. Excluding DTT reduced Z-RR-AMC catabolism (approx. 30%) when activity was assayed using a standard cathepsin B activity assay. DTT can be reduced to 0.1 mM with only a 10% decrease in Z-RR-AMC catabolism. Another alternative is to pre-activate the lysosomal enzymes with a small amount of reducing agent prior to the dilution of the lysosomes. Pre-activation can be achieved by buffering a small amount of the undiluted lysosomes with 10x buffer containing 2.0-2.4 mM DTT and incubating the mixture at 37°C for 10 minutes with shaking. After activation, the lysosomes should be diluted to the appropriate protein concentration and use in catabolic assays without any additional reducing agents. Pre-activation of catabolic enzymes has been described in the scientific literature (Arunachalam et al., 2000); pre-activation experiments have not been evaluated at Sekisui XenoTech.

6) Regardless of the matrix used for catabolism studies, the proper controls should be carried out to ensure that the compound/biologic does not have any unexpected instability due to the buffer conditions needed to simulate the lysosomal environment. This can be achieved by incubating the target in a buffered reaction in the absence of the lysosomes in parallel to the experimental reactions.

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