Recommended protocol

In-solution antibody-based assays

Introduction

Protein analyses in complex biological matrices demand specific and sensitive techniques such as antibody-based methods. However, *ex-vivo* degradation by active enzymes can compromise these methods by producing protein fragments unless the sample is stabilized rapidly post sampling. When performing studies using antibody-based assays in solution, *e.g.* ELISA, with stabilized samples, it is important to homogenize each sample thoroughly. Consideration must also be taken to the selection of extraction buffer which should be strongly denaturing. The best results are found when extraction is performed in denaturing buffers and the buffer-to-sample ratio is greater than 10, *i.e.* at least 10 µl of buffer for each mg of sample.

The following protocol has been tested for successful results with stabilized samples. However, other protocols may also work but should be tested for compatibility with the Stabilizor system and downstream assays. The protocol is primarily written with tissue samples in mind but a majority of the steps is also valid for other sample types.

Specific protocol

Sample collection

1. Make sure everything is ready for stabilization prior to sampling
   Extract sample and place in Maintainor Tissue card.
2. Immediately stabilize in Stabilizor instrument according to Stabilizor system manual using the appropriate program mode.
   Alternatively, the sample can be frozen and stabilized at a later stage.
3. If necessary, sub-dissect samples after stabilization but before freezing.
4. If you do not intend to analyze the samples directly, store samples at -80°C.
5. Weigh samples together with pre-weighed centrifugation tubes. If you intend to use pestles and grinding for sample homogenization, avoid collecting samples larger than 50 mg per 1.5 ml tube, because of the risk of sample loss during open-tube homogenization.
6. Calculate sample weight.

Extraction buffer

Stabilized samples should be extracted in denaturing buffers. ELISA assays samples should be extracted in a urea-based buffer, e.g. 8M urea in ddH2O, 2%. CHAPS or 0.5% Triton X-100 may be added if extraction levels are unsatisfactory. Add at least 10 µl of buffer for each mg of sample. For samples less than 10 mg use at least 100 µl of buffer.

Homogenization

Stabilized samples generally homogenize well but it is very important that the samples are thoroughly homogenized to facilitate re-solubilization of proteins. Soft tissue such as brain can be homogenized using a microtip sonication rod whereas firmer tissues such as liver or heart need a more rigid homogenization method such as sample grinding or ball mill pulverization. The combination of physical homogenization followed by rod sonication is recommended for best extraction.

Sample grinding using pestle for 1.5 ml tube

1. Use a pestle directly in an ordinary 1.5 ml centrifugation tube without the abrasive material (if any). It is commonly included abrasive material to sample grinding kits; however, this does not increase extraction efficiency for stabilized tissue samples.
2. Crush sample coarsely prior to adding extraction buffer.
3. Add 10 µl of extraction buffer for each mg of sample. Add the buffer in two steps and homogenize after each addition. Start by adding 30% of intended buffer and after initial homogenization, add the remaining buffer and continue homogenization.
4. Homogenize for at least 2 minutes using pestle; continue even when no traces of coarse material remain.
5. An additional sonication step is recommended to ensure complete solubilization of proteins.
6. Centrifuge at top speed for 25 min to sediment cellular debris.
7. Carefully recover supernatant into a new tube without disturbing the pellet.

Ball mill

1. Homogenization is preferentially done frozen, with tissue pieces smaller than 1 mm3. The tube holder and the sample tube, together with balls, should be cooled in liquid nitrogen prior to adding the sample.
2. Homogenize frozen sample for 1 min at maximum frequency.
3. Add 10 µl of extraction buffer for each mg of homogenized sample, while balls are still in tube. Mix while sample thaws and remove balls using a magnet when they are no longer covered with sample.
4. An additional sonication step is recommended to ensure complete solubilization of proteins.
5. Centrifuge at top speed for 25 min to sediment cellular debris.
6. Carefully recover supernatant into a new tube without disturbing the pellet.

Sonication

1. Using a microtip, sonicate at 40W output for at least 20 or until no traces of coarse material remain, preferably in pulses of 2 sec.
2. Centrifuge at top speed for 25 min to sediment cellular debris.
3. Carefully recover supernatant into a new tube without disturbing the pellet.

Pre-dilution

Due to the high concentration of urea in the extraction buffer, samples need to be diluted prior to analysis. For most ELISA based assays, a urea concentration below 1M will not generally affect the assay.

Dilute the samples with at least 8x volume using the sample dilution buffer recommended in the instructions for the intended assay. To avoid precipitation of proteins during dilution make a serial dilution in at least two steps, e.g. mix 1x volume of sample into 2x volume of dilution buffer. Subsequently, add buffer equal to 6x volume of the original sample. In some cases, protein dissolved in high concentration urea buffers can precipitate during storage in freezer. To avoid this, dilute the sample prior to freezing.

Protein quantification

Measure the concentration of protein using a urea-compatible kit, e.g. 2-D Quant kit (GE Healthcare). Protein quantification in more diluted urea (less than 1M) can generally be carried out in standard protein assays, e.g. BCA or Bradford.

Subsequent steps in the downstream analysis are not affected by the Stabilizor system and traditional protocols can be followed without alterations.