



## Estimation of collagen biosynthesis via [<sup>3</sup>H]-proline incorporation

Version 1.0

**Background** An estimation of collagen biosynthesis is often a useful phenotypic marker of cell function in vitro. Many cell types also show marked induction of collagen biosynthesis on treatment with TGF- $\beta$ . There are various methods available which allow collagen biosynthesis to be estimated, which have various advantages and disadvantages:

(a) measuring mRNA for collagen chains. This is an accurate and straightforward measurement, but there are over twenty collagen types and more than 40 gene products encoding them. Without pre-existing knowledge of which one(s) are made by a particular cell, it may be impracticable to measure them all. As with all mRNA measures, it does not demonstrate whether, or to what extent, collagen protein production is altered.

(b) directly measuring collagen protein. More typically, the N-terminal or C-terminal pro-peptides which are cleaved off the collagen chains during triple helix formation are assayed, usually by ELISA. This is accurate, quantitative and straightforward where an ELISA with the specificity for the desired collagen type and species already exists (e.g. for human type I collagen). For other collagens it is impractical to establish a de novo assay in most cases.

(c) estimate total collagen synthesis through [<sup>3</sup>H]-proline incorporation. We can exploit the fact that the collagen chains, with the repeating sequence (gly-X-pro)<sub>n</sub> contain much more proline per gram of protein than any other protein in the body. Consequently, an increase in total proline incorporation is an estimate of increased collagen biosynthesis, particularly if normalised to methionine incorporation as a measure of total protein synthesis. This method is only an indirect estimate, but it includes all collagen types synthesised by a particular cell, is robust across all species and is technically straightforward.

### Procedure

1. Subculture confluent cell population as for normal passaging (for example, divide a confluent flask of rat VSMCs at 1:3 dilution. Plate 0.5ml of cell suspension into 24 well plates. Prepare six wells per condition to be tested (for example, to test the effect of

5 concentrations of TGF- $\beta$  on collagen synthesis, you would need to prepare 36 wells; 6 for control and 6 each for the 5 different concentrations of TGF- $\beta$ ).

2. Incubate for 24hrs at 37°C. Check that the cells are attached and spread and look healthy under the microscope.
3. Make the additions to the various groups of 6 wells – for example, treat some of the wells with various concentrations of TGF- $\beta$ . Remember to treat the control cells with the appropriate vehicle (for example, 4mM HCl / 1mg/ml BSA for TGF- $\beta$  additions).
4. Incubate for a further 24hrs at 37°C. Check the cells are healthy under the microscope at the end of this incubation.
5. Add 0.8 $\mu$ Ci per well of [<sup>3</sup>H]-proline, without changing the culture medium to THREE of the wells in each group of six. Add 0.8 $\mu$ Ci/well of [<sup>35</sup>S]-methionine to the remaining three wells to act as an estimate of total protein synthesis used to normalise the proline incorporation.
6. Incubate for a final 24 hrs at 37°C. Once again, check the cells are in tact at the end of this incubation.
7. Wash the wells three times with ice-cold Dulbecco's PBS (do NOT use simple PBS). Remember that all washes will contain radioactivity.
8. Immediately on aspirating the final wash, add 0.5ml 1% (w/v) SDS to lyse the cells.
9. Add 0.5ml ice-cold 15% (w/v) trichloroacetic acid (TCA) solution and transfer the plate to a fridge at 4°C. This results in the TCA-mediated cold-precipitation of the macromolecular fraction (including all proteins) separating incorporated ligand for unincorporated ligand.
10. Scrape all wells carefully with a cell scraper.
11. Filter through GF/C filters on a vacuum manifold. Wash each well with 4x 1mL ice-cold 7.5% TCA and transfer the washings through the same filter. Wash the filter with 4ml of 96% ethanol to remove the TCA.
12. Air-dry the filters in the neck of scintillation vial inserts, then push into the inserts, add 4mL of scintillant, cap the inserts and count for <sup>3</sup>H or <sup>35</sup>S as appropriate. Report the mean and SEM of triplicate determinations. Express the impact of each treatment condition as the fold-change over control, normalised to the fold change in total protein synthesis under the same conditions.

### KEY POINTS:

- \* This protocol involves radioactivity. You must be registered and follow all local rules on safe working with radioactivity before using this protocol.
- \* Remember that all washings, at ALL steps of the protocol after step 5 (addition of the radioactivity) will be radioactive. Do not use the vacuum line aspirator for radioactive washes. Dispose of all radioactive material carefully. Swab all glassware after washing to confirm cleanliness. Record evidence of such swabbing in the radioactivity file.

\* TCA solution is a strong acid. The solid will cause severe burns if it comes into contact with naked skin. Use gloves and eye protection when handling. Mark bottles as "CAUTION: STRONG ACID".. Handle with extreme care throughout the procedure.

\* If required, improved incorporation fractions can be obtained by using DMEM deficient in proline and methionine, but this adaption is not generally recommended.

### *Materials*

L-[2,3- <sup>3</sup> H]-proline approx 50Ci/mmol	TRK638	Amersham Biosciences
L-[ <sup>35</sup> S]-methionine approx 1000 Ci/mmol	51001H	ICN Biomedicals

### Dulbecco's PBS

137mM NaCl (8g), 2.7mM KCl (0.1g), 8.1mM Disodium hydrogen phosphate (1.14g anhydrous), 1.5mM Potassium Dihydrogen phosphate (0.1g), 0.9mM Calcium chloride (900 $\mu$ l of 1M), 0.5mM Magnesium chloride (500 $\mu$ l of 1M).

Dissolve solids in 1 litre MilliQ, adjust to pH 7.2 if necessary.

### *Source References*

This protocol was adapted from our [<sup>3</sup>H]-thymidine incorporation assay for DNA synthesis.

### *References where we have used this protocol*

Grainger, D, Frow E. (2000) Biochem J. 350:291-298

### **DISCLAIMER**

**Please note that this protocol is provided for information only. While we believe it to be accurate, we do not warrant as to its validity or suitability for any particular purpose. In the event that you wish to use this protocol, we accept no liability for any direct or indirect losses howsoever caused. It is your responsibility to ensure that any use of this protocol is performed safely and in accordance with any local laws, regulations or guidelines applicable to you.**