Histone H4 Peptide Radiolabeling Protocol

Stock Solutions - **Prepare all solutions immediately prior to use:**

1. **[^{3}H]Acetic Acid (Sodium Salt):** 5mCi [H^3]-CH_3COONa in ethanol; 2-5Ci/mmol (DuPont-NEN Catalog #NET003).
2. **BOP solution** (0.24M BOP, 0.2M triethylamine in acetonitrile): Add 97µl of acetonitrile to 10mg of BOP and mix well to dissolve. Add 3µl of triethylamine to the solution and mix well.
3. **10mM HCl in methanol:** 4.3µl of concentrated HCl to 5ml of methanol.
4. **10mM HCl/10mM methanol:** Add 4.3µl of concentrated HCl and 2.0µl of methanol to 5ml distilled water.
5. **10mM HCl in 10% methanol:** Add 4.3µl of concentrated HCl in 4.5ml distilled water and 0.5ml of methanol.
6. **Elution Buffer** (3N HCl in 50% isopropanol): Add 1.29ml of HCl to 1.21ml of distilled water. Add 2.5ml of isopropanol.

Peptide Labeling Protocol:

Perform all procedures in a fume hood.
Always follow your institution's guidelines for proper use of radioisotopes.

Stage One: *Radiolabeling of Histone H4 Peptide*

1. Add 1.25mCi of [H^3]Acetic Acid (see Technical Note 1) to 100µg (one vial) of lyophilized **Histone H4 Peptide**. Mix thoroughly.
2. Add 5µl of the freshly prepared BOP Solution. Cap tightly, place mixture on rocker, and rock gently overnight at room temperature. This labeling mixture will be used in subsequent steps.

Stage Two: *Purification of Radiolabeled Peptide*

1. Place the **Microcon®-SCX Spin Column** into column support tube.
2. Prewash the column with 500µl of the 10mM HCl in methanol stock. Centrifuge for one minute at 2,000 x g. Repeat spin if necessary to ensure that all the liquid has been expelled from the column. Discard eluate.
3. Prewash the column with 500µl of the 10mM HCl/10mM methanol stock. Centrifuge for one minute at 2,000 x g. Repeat spin if necessary to ensure that all the liquid has been expelled from the column. Discard eluate.
4. Reserve an aliquot of labeling mixture from Stage One for calculation of percent [^{3}H] incorporation (see page 5). Load the labeling mixture into the spin column. Cap the top of the column with the support tube lid for this and all subsequent steps to prevent equipment contamination. Centrifuge at 1,200 x g for one minute.
5. Transfer the spin column to a fresh support tube.
6. Wash the spin column with 500µl of the 10mM HCl in 10% methanol stock. Centrifuge for one minute at 2,000 x g. Repeat spin if necessary to ensure that all the liquid has been expelled from the column.
7. Transfer the spin column to a fresh support tube. Discard the radioactive eluate from step 6 as radioactive waste.
8. Repeat steps 6 and 7.
9. Transfer inverted column to a fresh support tube. Inversion of the spin increases yield
10. Elute the radiolabeled peptide with 50µl of Elution Buffer stock. Centrifuge for 15 seconds at 14,000 x g.
11. Repeat steps 9 and 10 to ensure all the peptide has been recovered. Discard column and pool eluates. **Caution:** column filter can dislodge during second centrifugation.
12. Remove elution buffer from the radiolabeled peptide by using a speed-vac or by allowing the buffer to evaporate in a fume hood with the microfuge tube uncapped.
13. Dissolve radiolabeled peptide in 250µl of distilled water. Reserve an aliquot (~6µl) to determine purity of eluted peptide and percent [^{3}H]-acetate incorporation (see page 5).
14. Aliquot and store radiolabeled peptide at -70°C (see Technical Note 2).

I. Estimation of Radiolabeled Peptide Purity

A. Measure and calculate the average of three 1µl aliquots of the radiolabeled peptide from step 13 above. Serial dilutions of samples may be required to obtain an accurate measurement. Correct for any dilution as needed. The average equals the **total peptide CPM/µl** in the equation below.

B. Perform an ethyl acetate extraction to determine the extent of contamination with free [^{3}H]-acetate. Add 1µl of eluted peptide (step 13) to each of 3 tubes containing 100µl of distilled water. Add 400µl of ethyl acetate to each sample and vortex. Separate phases with a brief centrifugation, one minute at 14,000 x g, in microfuge.

C. Add 200µl of the upper, ethyl acetate phase and 50µl of the lower aqueous phase to separate scintillation vials containing 5ml scintillation cocktail. Measure the ethyl acetate extractable "Background" CPM (see Technical Note 3) and aqueous "Incorporated" CPM. Determine the average for each.

D. Determine the total-peptide to background ratio, which in a successful acetylation should be greater than 1000-fold over background (see Technical Note 4) using the following equation:

$$\frac{\text{total peptide CPM (step A)}}{\text{2 X Background CPM (step D)}}$$

II. Percent [^{3}H] Incorporation Calculation (see Technical Note 5)

A. Measure and calculate the average CPM/µl of the labeling mixture by counting three 1µl aliquots of labeling mixture (reserved material from step 4, page four).

B. Calculate % incorporated CPM eluted peptide using the following equation.

$$\frac{\text{2 X incorporated CPM (step I.C.) X eluted peptide, volume (µl) (step 13)}}{\text{labeling mixture CPM x labeling mixture, volume (µl) (step II.A.)}} \times 100$$

Technical Notes:

1. High specific activity [^{14}C]-acetic acid may be substituted [^{3}H]-acetate in this protocol.
2. 20,000CPM of radiolabeled peptide per 200µl HDAC assay is recommended. Radiolabeled peptide is stable for at least 1 year if stored at -70°C. Aliquots should be small enough to minimize repeated freeze/thaw cycles.
3. Ethyl acetate extracts any unincorporated [H^3]-acetic acid that may have co-eluted with radiolabeled peptide.
4. If the background is high, perform ethyl acetate extraction on the peptide sample (step 13) with four volumes of ethyl acetate. Vortex and centrifuge to separate phases, and recover the aqueous phase, avoiding the ethyl acetate. Repeat extraction until background (ethyl acetate extractable counts) is at least 1000-fold less than incorporated counts (aqueous phase).
5. Specific activity of radiolabeled peptide cannot be determined because amount of peptide recovered from spin column is unknown.