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Procedure for the Iodination of IGFs (Chloramine-T method)

Note that the operator should be trained in the safe use and handling of radioisotopes and hold an appropriate licence if required by the local radiation authority. Iodination must be performed in a laboratory equipped for safe handling of radioisotypes, containing a fume hood with exhaust and lead shielding.

Materials and Reagents

1. **Gro*Pep* IGF Peptide:** 1 x 100µg Receptor Grade IGF-I or IGF-II (lyophilized).

2. **Sodium Iodide $Na^{125}I$:** Supplied by GE Healthcare (Cat#IMS30)

3. **Chloramine-T:** Supplied by Sigma (Cat # C9887)

4. **Sodium Metabisulphite ($Na_2S_2O_5$):** Supplied by Sigma

5. **PD-10 Chromatography Column:** Supplied by GE Healthcare (Cat#17-0851-01)

6. **Chromatography buffer:** 50 mM sodium phosphate containing 150 mM sodium chloride and 0.25% (w/v) Bovine Serum Albumin (RIA Grade), pH 6.5

7. 0.5M Sodium Phosphate, pH 7.5

8. 10 mM HCl

9. 99% (w/v) Trichloracetic acid (TCA)

10. Laboratory Items: Tubes, racks and other general laboratory items.

Preparation of Reagents

1. **Gro*Pep* IGF Peptide** - Remove the metal cap from the vial and carefully loosen the bung to equalize the slight vacuum within the vial to prevent any loss of peptide upon opening.

2. Add 100µl of 10mM HCl acid to the vial, and gently vortex. Ensure that all the peptide is dissolved before proceeding.

3. Dispense 10µl aliquots into eppendorf tubes and store at -80°C. Aliquots will be stable for at least 1 year if stored at -80°C. Use for iodination when required.

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$Na^{125}I$: Supplied by GE Healthcare

1. Order $Na^{125}I$ from GE Healthcare (Cat# IMS30, approximately 100 mC_i/ml, 10 - 20 $mC_i/\mu g$)

2. Record batch / lot number, pH, reference date, activity and specific activity.

3. On the day of iodination, calculate the volume of $Na^{125}I$ to add to the iodination reaction given that 1 mC_i of radioactive label should be added. The reference date and the specific activity of the $Na^{125}I$ will affect the volume of solution that is required. (Note that the half-life of ^{125}I is 60 days. Refer to GE Healthcare (formerly Amersham Biosciences / Pharmacia Biotech), "Table of Decay of ^{125}I" included in the $Na^{125}I$ pack).

Chloramine-T (Sigma Cat # C9887)

1. Weigh out 0.1g Chloramine-T and place in a 10ml tube.

The following steps should only be completed immediately before commencement of the iodination reaction.

2. Add 10ml distilled or Milli-Q water and mix (resultant concentration is 10 mg/ml).

3. In a 10ml tube labelled as "Chloramine - T Working Solution", place 400µl of the 10mg/ml solution and dilute with 9.6ml of distilled or Milli-Q water.

4. Mix thoroughly. The "Chloramine - T Working Solution" is now ready for immediate use (0.4mg/ml solution). Discard all Chloramine - T solutions when the iodination procedure is completed.

0.5M Sodium Phosphate, pH 7.5

1. Weigh out 7.8g Sodium Monophosphate Dihydrate ($NaH_2PO_4.2H_2O$) and place in a 100ml beaker.

2. Add 90ml Milli-Q water to the beaker, mix to dissolve and adjust the pH to 7.5.

3. Adjust the volume to 100ml.

4. Prepare 0.5ml aliquots for convenient use in iodinations. Store at -20°C.

0.6 mg/ml Sodium Metabisulphite ($Na_2S_2O_5$)

1. Weigh out 0.1g of Sodium Metabisulphite and place in a 10ml tube.

***The following steps should only be completed immediately before commencing the iodination reaction*.**

2. Add 10ml distilled or Milli-Q water and mix (10mg/ml).

3. Place 600µl of the 10mg/ml solution in a 10ml tube and add 9.4ml of distilled or Milli-Q water. Label as "sodium metabisulphite solution" (0.6 mg/ml).

4. Mix thoroughly. The solution is now ready for use. Discard all metabisulphite solutions when the iodination procedure is completed.

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Chromatography buffer:
(50mM Sodium Phosphate buffer containing 150mM Sodium Chloride and 0.25% (w/v) BSA, pH 6.5)

To prepare 0.5 litre of chromatography buffer:
1. In a 500ml beaker, add 3.9g sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$), 4.383g NaCl and 1.25g BSA (Sigma Chemical Co., RIA Grade).

2. Adjust pH to 6.5 with 1M NaOH and adjust volume to 500ml with Milli-Q water.

3. Prepare a 20% (w/v) Sodium Azide (NaN_3) solution. In a fume hood add 20g NaN_3 to 100ml of Milli-Q water.

4. Add 0.5ml of the 20% (w/v) NaN_3 solution to the prepared chromatography buffer. The final concentration of NaN_3 in the buffer is therefore approximately 2% (w/v). The buffer is now ready for use.

PD-10 Column Preparation

1. Cut the bottom off the PD-10 column, place cover over the end and clamp into the retort stand.

2. Remove the top cap and pipette off the preserving solution. Equilibrate the column by completely filling the reservoir with Chromatography Buffer and removing the bottom cover to allow buffer to flow into column. It is suggested that a minimum of five column volumes are run through the column to block all non-specific sites on the column.

Procedures (All materials should be equilibrated at room temperature before use)

Iodination Reaction

Please note that this iodination procedure is designed to label GroPep IGF peptides to a specific activity of between 50 - 80 C_i/g. Note also that the iodination reaction must be completed in a Type B laboratory or certified iodination laboratory in a fume hood.

1. Add 50µl of 10mM HCl to a 10µg lyophilized aliquot of IGF peptide and let stand for 30 minutes at room temperature. Mix gently. The tube containing the reconstituted peptide is now referred to as the "iodination reaction tube".

2. Add 50µl 0.5M sodium phosphate pH 7.5 to the iodination reaction tube.

3. Add approximately 1 mC_i $Na^{125}I$ (approximately 10µl if the preparation is fresh) to the iodination reaction tube.

4. Add 20µl of 0.4mg/ml Chloramine-T Working Solution and start the timer.

5. Gently mix the contents of the iodination reaction tube.

6. Stand for 60 seconds only.

7. Add 20µl 0.6 mg/ml sodium metabisulphite to the iodination tube; mix gently. Stand the tube for 5 minutes.

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Isolation of [^{125}I] - labelled IGF from unreacted Na^{125}I and other reaction products.

1. Dilute the iodination reaction mixture with 300µl chromatography buffer.

2. Load onto the equilibrated PD-10 column and allow the reaction mixture to run onto the column. Further wash reaction mixture into the column with 3 x 500µl of chromatography buffer to allow the sample to enter the column bed. **This washing-in step is important!**

3. Collect 60 x 30 second fractions into 4DT tubes. Top up the column reservoir whilst collecting fractions to ensure the column does not dry out.

Selection of the fraction containing the desired [^{125}I] - labelled IGF peptide

The fractions which contain the [^{125}1] - labelled IGF peptide and whose radioactivity is greater than 95% TCA precipitable are chosen for use.

A. Plot the gamma radioactivity per 5µl of each fraction versus the fraction number.

1. Label a set of 4DT of tubes equal to the number of fractions collected. Add a 5µl sample from each fraction to the corresponding numbered tube. Dilute with 900µl of chromatography buffer.

2. Count each diluted sample tube for gamma radioactivity.

3. Plot the data on a graph showing the gamma radioactivity per 5µl of each fraction number.

B. Determine the percentage of TCA precipitable radioactivity contained in each fraction.

1. After counting the diluted sample tubes, add 100µl of 99% (w/v) TCA to each tube. Vortex and let stand on ice for a minimum of 30 minutes to maximize protein precipitation.

2. Centrifuge for 10 minutes at 4,000 g.

3. Remove the supernatant from each test tube, and transfer to another series of labelled test tubes and count the supernatant fraction for gamma radioactivity.

4. The percentage of each fraction that is TCA precipitable is the ratio between the radioactivity contained in the pellet and the sum of the radioactivity in the pellet plus the supernatant. This is termed the percent TCA - precipitable.

5. Plot the TCA - precipitable radioactivity percentage for each fraction on the same graph as the [^{125}I] gamma radioactivity.

6. The fractions whose radioactivity is greater than 95% TCA precipitable will contain the [^{125}I] - labelled IGF peptide. (Do not include the aggregate fraction which may be the first small peak of radioactivity seen).

7. Pool the chosen fractions and aliquot into convenient sized aliquots, commensurate with the planned use of tracer.

8. Store at -20°C. Avoid repeated freeze-thawing of aliquots.