

Dear Researcher,

Hynic-Annexin will be shipped to you frozen along with lyophilized stannous tricine. Tricine is a modified glycine used in the labeling of Hynic. The reagents should be stored in a  $-20^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$  freezer (preferentially  $-80^{\circ}\text{C}$ ).

The Hynic-Annexin vials are ready for radiolabeling with Tc-99m (Sodium Pertechnetate) as follows:

First remove the vials from the freezer at the time of preparation and allow them to thaw at room temperature (about 5 min). **Note: Please do not heat the vial.** Second, after thawing please add 30 to 50 mCi (0.5 ml) of Pertechnetate to the annexin vial and gently swirl. Then, reconstitute a stannous tricine vial with 3.0 mL of saline for injection followed by immediate removal of 300  $\mu\text{L}$  (about 50  $\mu\text{g}$ ), which you will add to the Pertechnetate/Hynic-Annexin vial. Swirl the vial and incubate for 15 min. at room temperature, take the sample for quality control, then the preparation is ready for further dilution. Please note that the stannous tricine solution expires at the end of the day (12 hr stability), so, it must be prepared at the time of the labeling.

The radiolabeling yields are greater than 90% in as short of a time as 15 min. Quantization can be performed using ITLC-SG strips developed in ACD buffer. The amount of activity that stays at the origin of the plate is counted and compared to the total plate activity.

Attached is additional information, generously provided by Dr. Jean-Luc Vanderheyden (jlvand@earthlink.net), and concerning the labeling procedures and answers to some of the typical questions related to the radiolabeling.

Please contact the BRB-Preclinical Repository if you have any questions.

Good luck with your research,

Karen Muszynski

Karen Muszynski, Ph.D.  
Director, BRB Preclinical Repository  
Developmental Therapeutics Program  
DCTD, NCI, NIH  
Email: muszynskik@mail.nih.gov

### **Additional questions related to Tc-99m Hynic-Annexin radiolabeling conditions:**

*Q1 - Hynic-annexin V is supplied 0.275 mg/vial. I cannot use all the product, since I only need 5 ug/kg annexin V for mice imaging, and I don't have excess Tc-99m. Could one divide one vial to several aliquots?*

Yes, you can sub-divide the Hynic-Annexin but you cannot do this for the stannous tricine. Here is the recommended protocol:

1. Thaw a vial of Hynic-Annexin at room temperature. It will only take 5-8 minutes.
2. Open the vial by uncrimping the aluminum cover. If you don't have a decrimper for that size, you can use a pair of scissors, and place one blade under the lip of the aluminum foil and the other on top of the vial. After a few trials, you should be able to get the aluminum only, leaving the stopper sealed on the vial.
3. Have prepared tubes (the ones in which you will radiolabel), preferentially 1.5 mL conical (either screw top or cap), which you will have labeled properly with date, concentration and storage conditions.
4. Dispense the amount that you wish to aliquot. We have tested 25 ug Hynic-Annexin before. Make sure that your tube is upright, closed and place in a -80°C freezer immediately. It is important that you do these steps quickly in order to avoid decomposition of the Hynic and maintain high radiochemical purity.
5. When you are ready to perform a labeling, take one of these vials, and thaw. There is enough tricine in the Hynic-Annexin vial to support good radiolabeling yields even if you don't use the stannous tricine.

*Q2 - About the stannous tricine solution, is it possible to aliquot them since it seems that the reconstituted solution is only stable only for one hour or so?*

The reconstituted stannous tricine cannot be easily aliquoted. We, and our collaborators, have found that it yielded significant amounts of TcO<sub>2</sub>. Note that the purpose of the stannous tricine is to be a source of stannous ions for radiolabeling. Therefore, you can make your own stock solution of stannous chloride dihydrate (1 mg/mL) in 0.05 M HCl and use that instead. Of course, similarly, the stannous chloride solution is decomposing with time, but you should have a good supply of stannous and diluted HCl. The solution is good for a little longer than 1 hr, and has been used for ½ day, but you will see that the tin will come out of solution after that period of time.

*Q3 - If one Hynic-Annexin vial is divided into several aliquots, vials, how does one change the condition of labeling?*

The radiolabeling needs to be adjusted accordingly. You should not attempt to do radiolabeling with Tc-99m at greater than 200 uCi/ug. Therefore, you will be limited by what you can use in your experiments to about 5 mCi Tc-99m. If your elution is "old", then it will be worse (as it will be quite a bit more of Tc-99g). You should use Tc-99m that has been eluted within about 4 hr. Also, don't use "first generator eluant" as there has been impact on stability of Tc-99m Hynic-Annexin.

The radiolabeling procedure would then be as follows:

1. Thaw the Hynic-Annexin aliquot.
2. Add about 200 uL saline (to give it some volume; this is important so that the stannous concentration is diluted)

3. Add the pertechnetate (we suggest 10 to 30 uL)
4. Add the stannous solution, about 20ug maximum, either from the stannous tricine or from the extemporaneously prepared stannous/HCl.
5. Mix and cap, wait 15 min.

Important steps: if you see a precipitate or cloudiness, it is likely that the pH is too acid. Therefore, please check the pH at the beginning to ensure that it is greater than 5.0. Recommended pH is between 5.5 and 6.5. Failure to check the pH may result in the denaturation of the protein.

*Q4 – Regarding quality control, ITLC and ACD buffer: is it the acid citrate dextrose (68mmol/L dextrose, 74mmol/L citrate, pH 5.0)? In certain publications saline was used for RCP. Can I use saline?*

For the ITLC SG, you should activate plates in an 110°C oven for 30 min. as recommended by the manufacturer. It works also with "unactivated" plates, but the results are not always very tight (+/- 2% or so). ACD can be purchased from Sigma/Aldrich (Catalog #C3821), or you can make it yourself. It is not very expensive and not much is required. Saline has been used in the past, but does not allow for the differentiation of hydrolyzed/reduced TcO<sub>2</sub> that may be formed in the product. With ACD, Verbeke et al reported that TcO<sub>2</sub> would likely move on the plate.

Reference: Verbeke K, Kieffer D, Vanderheyden JL, Reutelingsperger C, Steinmetz ND, Green AM, Verbruggen A. Optimization of the preparation of (99m)Tc-labeled Hynic-derivatized Annexin V for human use. 2003. Nucl Med Biol. 30:771-778.

Note: The Radiochemical purity (RCP) should be greater than 90%. It is typically 94-95%.

*Q5- How much Pertechnetate activity can be added per vial?*

The critical factor is the total amount of Tc-99g that is contained in the Pertechnetate solution; the older the pertechnetate, the lower its specific activity. We recommend keeping the volume of pertechnetate low, at 0.5 mL. We recommend you do not use the first generator eluant as it may contain oxidants that interfere with the radiolabeling process.

*Q6- How long does the Hynic-Annexin vial remain stable?*

We have about 12-month stability at -20°C and over 36 months at -80°C. The glass transition temperature for the Hynic-Annexin product is about -45°C, so, any temperature below that will have increased stability.

*Q7 - How long does the Tc-99m Hynic-Annexin remain stable?*

When the Tc-99m Hynic-Annexin is prepared in presence of stannous ions and tricine it is stable for about 6 h. To avoid degradation, we recommend performing dilution and storage of the product in the reaction vial until time of use.

*Q8 - Can the solution be filtered by a 0.22 um filter?*

It is recommended that you filter the solution through a 0.22 um filter and measure the activity loss. For animal studies, a Gelman Acrodisk (13 mm) has been used successfully. These are low protein binding, and have worked well.

*Q9 - Can the solution be diluted with i.e. NaCl 0.9% and are there minimum/maximum dilutions?*

Yes. Once you have done QC testing and you are ready to proceed, you can dilute with NaCl 0.9%. There has been a noticeable loss of radioactivity by adsorption to the glass vial in cases where the solution was diluted below 5 ug/mL. This did not seem to affect the purity, but about 25% of the activity was "hanging" on the vial. Also, it is recommended that you adjust the amount of activity at the time of the preparation so that the product is not diluted any further than 10-fold, or you may add albumin as part of the diluent.

*Q10 - Do you have a procedure for quality control (RC-purity)?*

Yes, there are two methods. If only one method is available to you, that's OK. ITLC is used to determine overall radiochemical yields, and HPLC to determine radiochemical purity.

Note that further purification is not needed, nor is it necessary. However, if you wish to further process the sample, the preparation can be placed on a conditioned PD-10 (Sephadex G-25) column, and eluted in PBS. It is better if the PBS is made to contain about 2 mg/mL of tricine and to use the material quickly after purification.

#### **For the radiochemical yields by ITLC:**

The following must be prepared and assembled in advance.

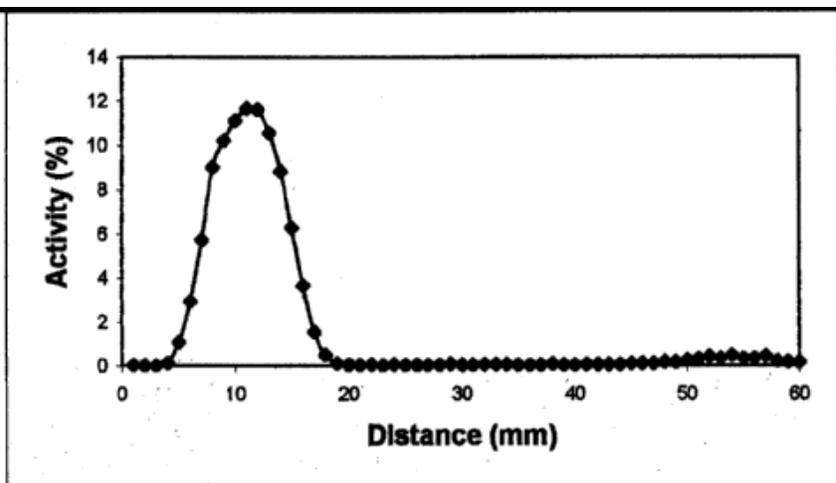
1. Thin layer chromatography developing chamber
2. Dose calibrator, gamma counter or appropriately calibrated radioTLC scanner
3. Chromatographic Solvent: Place a small amount of ACD buffer [QC Component 1] in the chamber in order to have solvent at about the 0.5 cm level above the bottom of the chamber.
4. Silica Gel Impregnated Glass Fiber Sheets (Gelman): Pre-cut the strips to a final dimension of approximately 2 cm wide by 6 to 9 cm in height. Activate the plates in an 110°C oven for 30 min before use; then store in a glass jar with a screw cap. The plates are OK for 1-2 day use, so, you need to reactivate for a following day.

**NOTE: THE STRIPS ARE FRAGILE. USE FORCEPS FOR HANDLING.**

#### **Test Procedure**

1. Carefully remove an ITLC chromatographic strip from a storage container using forceps. Using a pencil, carefully mark the origin at approximately 1 cm from one end of the strip.
2. Spot a small drop (2 to 5µl) of product at the origin of the ITLC chromatographic strip.
3. Place the chromatographic strip into the developing chamber. Do not immerse the spot into the solvent.
4. Develop the chromatographic strip, allowing the solvent to ascend to about 0.5 cm from the strip top. Remove the strip from the developing chamber and allow it to dry.
5. Place the strip on a clean sheet of paper and either use a calibrated radioTLC Scanner or cut the developed chromatographic strip into three sections.

An example of radioTLC chromatogram is attached below. Integration is accomplished by totaling the activity of the peak at the origin over that of the whole strip, and using the formula as described in step 8.



6. If cutting is chosen, identify the sections as solvent front area (a), middle area (b), and origin area (c).

Section name	6 cm strip	9 cm strip
Origin area (c)	0 to 2.75 cm	0 to 4.25 cm
Middle area (b)	2.75 to 3.25 cm	4.25 to 4.75 cm
Solvent front area (a)	3.25 to 6 cm	4.75 to 9 cm

7. Using the developing system described above, Technetium Tc-99m Hynic-rh-Annexin-V remains at the origin and non-protein bound Technetium Tc-99m labeled material travel with the solvent front. The middle section of the strip is used to verify complete separation between product and impurities (less than 5% of the total Tc-99m activity should be in this section of the strip). **If the middle section is greater than 5%, please repeat the testing in duplicates and report all results.**
8. Using a suitable radioactivity counter (e.g., dose calibrator or gamma counter), count each section of the strip. Count long enough to determine a statistically significant count or count rate for each strip section. Calculate the radiochemical purity (percent Technetium Tc-99m Hynic-rh-Annexin-V) using the following formula:

$$\% \text{ radiochemical purity} = [c/(a + b + c)] \times 100$$

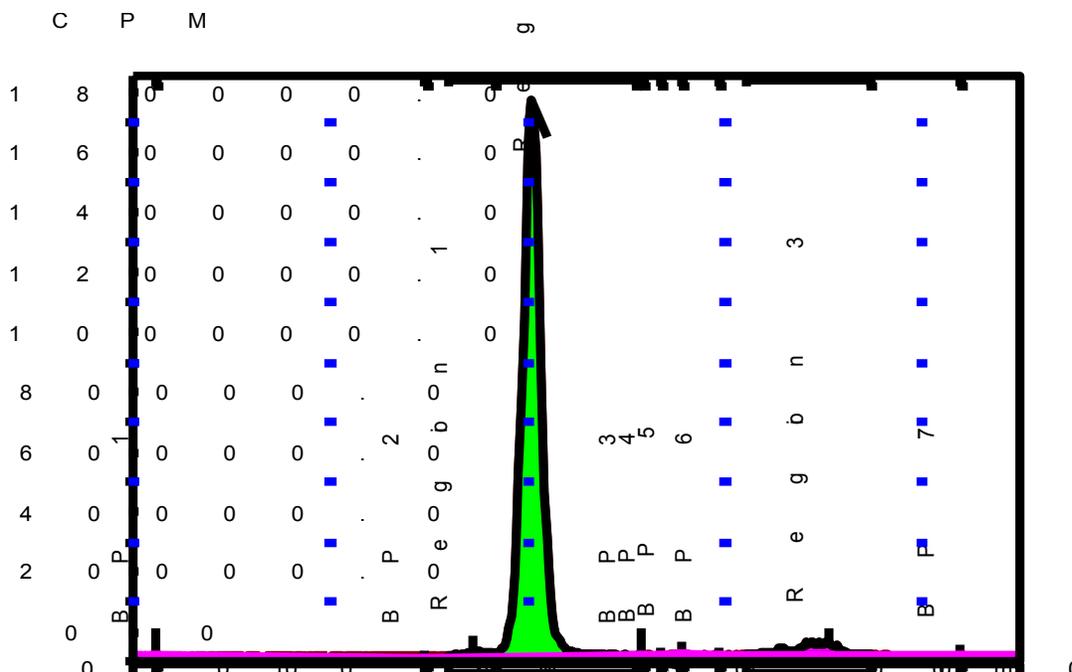
**For the radiochemical purity by HPLC:** use a Superose column (see typical result below). The UV trace is uninformative, so only the radioactive trace is provided.

Superdex 75 HR 10/30 column (Pharmacia)

0.05 M Phosphate buffer pH 7.0 containing 0.15 M NaCl and 1 mM sodium azide

Flow rate: 0.5 mL/min, about 200 psi, column at room temperature

Gamma detection: PE FSA Analyzer



Name (Tc-99m)	Start (mins)	End (mins)	Retention (mins)	Height (CPM)	Area %
BP 1	1.10	1.20	1.10	1685.7	
BP 2	14.80	14.90	14.80	1528.6	
Region 1	16.00	18.40	17.30	4114.3	1.37
Region 2	18.50	25.50	20.20	187957.1	92.93
BP 3	25.80	25.90	25.80	1671.4	
BP 4	26.80	26.90	26.80	2071.4	
BP 5	27.80	27.90	27.80	2314.3	
BP 6	29.70	29.80	29.70	2142.9	
Region 3	31.10	37.40	35.30	6700.0	5.70
BP 7	42.00	42.10	42.00	1800.0	
3 Peaks					100.00
Total Area					
Background					1764.9
Unallocated Area					

*Q11 - I have a large animal to inject, can I combine vials?*

You can combine vials, just prior to labeling, by transferring research material into a larger reaction vial, or, after radiolabeling.

*Q12 - What other tests might be important to perform?*

A visual check of the product is recommended. Also, pH should be measured. The pH is typically at pH 6 (between 5 and 7).

**DETERMINATION OF pH**

**Test Procedure**

1. Remove a pH strip from its container.
2. Spot a small drop of product on the three sections of the pH paper.

3. Within 5 minutes, compare the color of the strip with that of the color chart on the box in order to determine the pH of the product.

Record results of the pH measurement.

**If the pH is less than 5 or greater than 7 DO NOT administer the product.**

### *VISUAL ASSAY*

#### **Test Procedure**

1. Swirl the vial in an upright position, and look across the diameter of the vial against a black background.
2. The solution must be clear, colorless immediately after reconstitution, and may present slight flocculation over time. Such flocculation does not affect the quality of the preparation nor preclude its preclinical use since the preparation is filtered prior to injection.