Radioimmunoassay for the quantitative determination of Angiotensin I/Plasma Renin Activity in human plasma.

**REF**

**SO19011**

**Σ**

100

For illustrative purposes only.
To perform the assay the instructions for use provided with the kit have to be used.
1. INTRODUCTION

Renin, a proteolytic enzyme with a molecular weight of approx. 40,000 daltons, is released from the juxtaglomerular cells of the kidney. This enzyme cleaves to its substrate, angiotensinogen, forming a decapeptide, angiotensin I, of molecular weight approx. 1,300 daltons. This polypeptide is further hydrolyzed by the converting enzyme (ACE) into the biologically active octapeptide angiotensin II.

The hormonal product of the renin-angiotensin system, angiotensin II, has an extremely short in vivo half-life, but it is the most potent vasopressor known; its main effects are vasoconstriction, stimulation of the sympathetic nervous system and stimulation of aldosterone secretion by the adrenal glands.

Since angiotensin I levels are a direct representation of plasma renin activity, the determination of plasma renin activity has been widely adopted to evaluate the renin-angiotensin system in disease states. Measurement of plasma renin activity (PRA) in hypertensives is an important aid in the differential diagnosis of primary and secondary aldosteronism.

Different factors influence renin secretion, which is inhibited by plasma levels of angiotensin II and ADH, by increased sodium and potassium retention and by increased renal perfusion pressure. On the contrary, sodium and potassium depletion, a reduced renal perfusion pressure and the activity of sympathetic nervous system, all result in an increased renin secretion.

2. PRINCIPLE OF THE ASSAY

The measurement of renin activity consists of radioimmunoassay of angiotensin I. The main steps of the assay procedure are:

- generation of angiotensin I in plasma samples during an incubation at 37°C under conditions which prevent the degradation of angiotensin I (presence of enzymatic inhibitor PMSF) and are considered most suitable for renin activity
- angiotensin I coated-tube radioimmunoassay in two aliquots of the same sample, one incubated at 37°C for generation and one non-incubated (sample blank).

The principle of the radioimmunoassay is based on the competition between labelled angiotensin I and angiotensin I contained in calibrators or samples to be assayed for a fixed and limited number of antibody binding sites. After the RIA incubation, the amount of labelled angiotensin I bound to the antibody coated on the tube walls is inversely related to the concentration of unlabelled angiotensin I present in calibrators or samples. The method adopted for B/F separation is based on the use of antibody-coated tubes, where the antibody is coated on the tube walls.

3. REAGENTS PROVIDED IN THE KIT

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coated tubes</td>
<td>100</td>
</tr>
<tr>
<td>125I-labelled angiotensin I</td>
<td>2 vials</td>
</tr>
<tr>
<td>Angiotensin I calibrators</td>
<td>6 vials</td>
</tr>
<tr>
<td>Control plasma</td>
<td>1 vial</td>
</tr>
<tr>
<td>Generation buffer</td>
<td>1 vial</td>
</tr>
<tr>
<td>Enzymatic inhibitor (PMSF)</td>
<td>1 vial</td>
</tr>
<tr>
<td>Number of tubes</td>
<td>100</td>
</tr>
</tbody>
</table>

STORAGE: Upon receipt, the kit should be stored at 2-8°C. Do not freeze. Once opened, the reagents of this kit are stable until the kit expiry date when properly stored. The kit has been designed to perform 3 assay runs when reagents are stored as the manufacturer recommends.
Reagents should not be used past the expiry date. The expiry date of the kit is reported on the external label and corresponds to the expiry date of the tracer. The expiry date of each component is reported on the respective vial label.

When reconstituting the contents of the vials, mix gently to avoid foaming.

Reagents from different batches must not be mixed.

3.1. Coated tubes
The inner surface of each tube is coated with biotinylated angiotensin I IgG raised in rabbits.

Before use, bring the coated tubes to room temperature prior to opening the box, to avoid condensation of humidity. Securely reseal the box containing unused tubes. Do not mix different batches of coated tubes.

3.2. 125I-labelled ileu-5-angiotensin I (red): lyophilized reagent
Each vial contains the hormone labelled with 125I, BSA, phosphate buffer, stabilizers, preservatives and an inert red dye. Radioactivity is 81 kBq (2.2 µCi) or less per vial on the calibration date.

Reconstitute the contents of each vial with 26 mL distilled water. Store the resulting solution in deep-frozen aliquots (–20°C or below).

3.3. Ileu-5-angiotensin I calibrators: lyophilized reagent
The vials contain increasing amounts of angiotensin I, BSA, phosphate buffer and preservatives.

Reconstitute the contents of each vial with 1 mL distilled water. The resulting solutions contain 0 - 0.3 - 1 - 3 - 10 - 50 ng/mL respectively and should be stored in deep-frozen aliquots (–20°C or below).

The kit calibrators demonstrate commutability with patient samples when used with reagents and operating procedure of this in vitro diagnostic test as the manufacturer recommends.

3.4. Control plasma: lyophilized reagent
The vial contains human plasma and preservatives. The reference range is reported on the vial label.

Reconstitute the vial contents with 2 mL chilled distilled water, taking care that the temperature does not exceed 4°C. Store the resulting solution in deep-frozen aliquots (–20°C or below).

The control plasma must be treated as a specimen and angiotensin I should be generated for 90 min at 37°C before assaying.

3.5. Generation buffer (blue): ready-to-use reagent
The vial contains 4 mL citrate buffer solution, stabilizers, preservatives and an inert blue dye.

3.6. Enzymatic inhibitor (PMSF): ready-to-use reagent
The vial contains 0.5 mL 5% phenylmethylsulphonyl fluoride (PMSF) (R 23/24/25, S 36, S 45) ethanol solution (R 11, S 7, S 16).

If crystallization occurs at 2-8°C, warm the PMSF solution to 37°C.

4. EQUIPMENT AND MATERIALS REQUIRED, BUT NOT SUPPLIED
- Distilled or deionized water.
- Glassware.
- Disposable polystyrene tubes.
- Test tube rack.
- Micropipettes with disposable tips (10, 50, 200, 500 µL) (10, 50 µL: trueness ± 3%, precision 2%; 200, 500 µL: trueness ± 2%, precision 1%).
- Vortex mixer.
- Thermostatically-controlled water bath capable of maintaining 37° ± 1°C.
- Device for aspiration of incubation mixture.
- Gamma counter suitable for counting 125I (counter window setting: 15-80 keV - counter efficiency: 70% - counting time: 1 min). If counter efficiency is below 60%, counting time should be prolonged to 2 min.
5. SPECIMEN COLLECTION AND PREPARATION

Specimen collection
Careful standardization of the patient preparation and sampling conditions is strongly recommended. Blood should be collected in pre-chilled tubes with sodium EDTA as an anticoagulant. The blood samples must not be withdrawn using heparin as an anticoagulant, as it interferes with angiotensin I generation. Conversely, EDTA offers the advantage of being a suitable anticoagulant, whilst simultaneously assisting in the inhibition of the converting enzyme. The samples must be kept cold and centrifuged in the cold at about 2000 X g* to recover the plasma. Samples having particulate matter, turbidity, lipaemia, or erythrocyte debris may require clarification by filtration or centrifugation before testing. Grossly haemolyzed or lipaemic samples as well as samples containing particulate matter or exhibiting obvious microbial contamination should not be tested. If the assay is not immediately performed, the samples should be aliquoted and stored deep-frozen (-20°C or below) until assayed. If samples are stored frozen, mix thawed samples well before testing, taking care that the temperature does not exceed 4°C. Avoid repeated freeze-thaw cycles.

Angiotensin I generation
Angiotensin I generation at pH 6.0 is performed under optimal conditions with subsequent better sensitivity for low renin activity samples and possible use of shorter generation times. The chosen generation conditions allow working with the lowest sample dilution possible thus reducing the effect of dilution on angiotensin I generation kinetics.

- Add the following reagents into non-coated generation tubes kept in an ice bath, strictly respecting this order:
  .  500 µL sample
  .  10 µL PMSF
  .  50 µL generation buffer.
- Mix the contents of tubes with a Vortex and transfer 200 µL of each sample into a second series of non-coated tubes.
- Incubate the second series of tubes for 90 min at 37°C in a thermostatically-controlled water bath, while keeping in an ice bath the first series of samples (sample blanks).
  The suggested generation time is the average time that allows PRA determination in many physiopathological conditions. When either higher or lower PRA values are expected, this time should be either reduced or prolonged (Fig. 1).
- After the 37°C incubation, the generation tubes must be immediately placed in an ice bath.

6. ASSAY PROCEDURE
Perform the assay at room temperature (20-25°C) at least in duplicate. Calibrators must be tested directly in the RIA procedure with coated tubes, without previously generating angiotensin I and must be run with each series of patient specimens. Calibrators and samples should be subjected to the same process and incubation time. Perform all assay steps in the order given and without any appreciable delays between the steps.
A clean, disposable tip should be used for dispensing each calibrator and sample.
- Dispense reagents in the bottom of coated tubes. Operate according to the following scheme:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Tubes</th>
<th>Calibrators 0-5</th>
<th>Samples &amp; blanks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrators</td>
<td>50 µL</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Samples</td>
<td>–</td>
<td>50 µL</td>
<td>–</td>
</tr>
<tr>
<td>Tracer</td>
<td>500 µL</td>
<td>500 µL</td>
<td></td>
</tr>
</tbody>
</table>

- Mix the contents of tubes with a Vortex and incubate for a time ranging from 3 to 24 hours at room temperature.
- Carefully aspirate the incubation mixture. Be sure that the aspirator tip touches the bottom of the coated tube so that all the liquid is removed. Failure to remove adhering solution adequately may result in poor reproducibility and spurious results. No trace of dye should still be visible.
- Measure the radioactivity of tubes.

\[ g = (1118 \times 10^{-8})(\text{radius in cm})(\text{rpm})^2 \]
7. CALCULATION OF RESULTS

Compute the mean net counts for each group of tubes. Compute the B/Bo ratio for each calibrator and unknown sample as follows:

\[
B/Bo\% = \frac{\text{calibrator or sample mean counts}}{\text{zero calibrator mean counts}} \times 100
\]

Plot in semilog coordinates the mean percent value for each calibrator on the ordinate (y axis) as a function of angiotensin I concentration expressed as ng/mL on the abscissa (x axis). A calibration curve is thus obtained (Fig. 2).

Plasma renin activity (PRA) is calculated as ng angiotensin I generated/mL/hour, following the procedure herebelow:
- directly from the calibration curve, read the angiotensin I concentration generated in each sample incubated at 37°C and in the respective sample blank (kept in an ice bath)
- subtract the corresponding blank value from each sample value
- multiply the obtained value by 1.12 as samples are initially diluted 1:1.12
- divide the concentration obtained by the generation time (hours):

\[
PRA = \frac{(\text{ng 37°C} - \text{ng 4°C}) \times 1.12}{\text{hours of incubation}} = \text{ng/mL/hour}.
\]

If generation time lasted 1.5 hours, equation (1) may be so simplified:

\[
PRA = (\text{ng 37°C} - \text{ng 4°C}) \times 0.747 = \text{ng/mL/hour}.
\]

**Fig. 1** - Check of the linearity of angiotensin I generation with increasing incubation time at 37°C, pH 6.0.

**Fig. 2**

\[
y = 9.78 \times + 2.44 \quad r = 0.993
\]

\[
y = 2.52 \times + 0.48 \quad r = 0.994
\]
Calculation example

The following data must only be considered an example and should not be employed instead of the data obtained by the user.

<table>
<thead>
<tr>
<th>Description</th>
<th>cpm</th>
<th>B/Bo x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero calibrator</td>
<td>19,230</td>
<td>100</td>
</tr>
<tr>
<td>0.3 ng/mL</td>
<td>17,692</td>
<td>92.0</td>
</tr>
<tr>
<td>1 ng/mL</td>
<td>14,519</td>
<td>75.5</td>
</tr>
<tr>
<td>3 ng/mL</td>
<td>9,807</td>
<td>51.0</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>5,000</td>
<td>26.0</td>
</tr>
<tr>
<td>50 ng/mL</td>
<td>2,115</td>
<td>11.0</td>
</tr>
<tr>
<td>Sample blank</td>
<td>17,499</td>
<td>91.0</td>
</tr>
<tr>
<td>Sample</td>
<td>9,038</td>
<td>47.0</td>
</tr>
</tbody>
</table>

By interpolation from the calibration curve and using equation (1), the sample is found to contain a plasma renin activity of 2.39 ng/mL/hour.

\[
PRA = \frac{(3.5 - 0.3) \times 1.12}{1.5} = 2.39 \text{ ng/mL/hour.}
\]

8. EXPECTED VALUES

The ranges given below are merely indicative for a sodium intake of 100-150 mEq/24 hours; each laboratory should establish its own reference ranges.

<table>
<thead>
<tr>
<th>Generation at pH 6.0</th>
<th>Supine position</th>
<th>Upright position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA, ng/mL/hour</td>
<td>0.2 - 2.8</td>
<td>1.5 - 5.7</td>
</tr>
</tbody>
</table>

9. SPECIFIC PERFORMANCE CHARACTERISTICS

9.1. Analytical specificity

Analytical specificity may be defined as the ability of the assay to accurately detect specific analyte in the presence of potentially interfering factors in the sample matrix (e.g., haemolysis, effects of sample treatment), or cross-reactive analytes.

Since in this particular case the amount of substance to be assayed is generated in vitro and the sample blank is subtracted, the actual specificity of angiotensin I antibody is not critical. However, rather than cross-reactions with circulating angiotensin-related peptides, the interference of sample proteins (sample blank) can set some limitations to the assay sensitivity. The overall sample blank includes the contributions of angiotensin-like proteins, circulating angiotensin I and angiotensin I produced by the renin action at low temperature from the time of blood collection to the assay.

**Interference.** Controlled studies of potentially interfering substances or conditions showed that the assay performance was not affected by lipaemia (up to 500 mg/dL triglycerides), bilirubinaemia (up to 20 mg/dL bilirubin). Angiotensin I values determined in samples containing 1000 mg/dL haemoglobin are about 10% lower than those determined in normal samples.

**Cross-reactions.** The percentage of cross-reactions by some angiotensin-related peptides, calculated according to Abraham, shows the specificity of the antibody used.
- Angiotensin I 100%
- Angiotensin II < 0.1%
- Heptapeptide, hexapeptide << 0.02%
9.2. Analytical sensitivity
Analytical sensitivity may also be expressed as the limit of detection, which is the minimal amount of specific analyte detectable by the assay. The limit of detection is 0.20 ng/mL at 95% confidence limit. This was calculated as the apparent concentration of analyte which was distinguishable from the zero calibrator, that is, two standard deviations below zero.

9.3. Precision
Different sample pools, at different PRA values, were assayed to determine repeatability and reproducibility of the assay (i.e., within- and between-assay variability).

<table>
<thead>
<tr>
<th>Repeatability</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of determinations</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (ng/mL/hour)</td>
<td>2.3</td>
<td>8.8</td>
<td>13.5</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.17</td>
<td>0.48</td>
<td>1.34</td>
</tr>
<tr>
<td>Coefficient of variation (%)</td>
<td>7.5</td>
<td>5.4</td>
<td>9.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reproducibility</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of determinations</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (ng/mL/hour)</td>
<td>2.6</td>
<td>8.6</td>
<td>13.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.20</td>
<td>0.70</td>
<td>1.50</td>
</tr>
<tr>
<td>Coefficient of variation (%)</td>
<td>7.7</td>
<td>8.1</td>
<td>11.5</td>
</tr>
</tbody>
</table>

9.4. Trueness
The assay trueness has been checked by the dilution and recovery tests.

**Dilution test.** Two plasma samples with high angiotensin I concentration were tested after serially diluting with the zero calibrator.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Expected concentration, ng/mL</th>
<th>Measured concentration, ng/mL</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>neat</td>
<td>–</td>
<td>17.5</td>
<td>–</td>
</tr>
<tr>
<td>1:2</td>
<td>8.75</td>
<td>8.6</td>
<td>98.3</td>
</tr>
<tr>
<td>1:4</td>
<td>4.38</td>
<td>4.5</td>
<td>102.9</td>
</tr>
<tr>
<td>1:8</td>
<td>2.19</td>
<td>2.3</td>
<td>105.1</td>
</tr>
<tr>
<td>1:16</td>
<td>1.09</td>
<td>1.1</td>
<td>100.6</td>
</tr>
<tr>
<td>neat</td>
<td>–</td>
<td>8.5</td>
<td>–</td>
</tr>
<tr>
<td>1:2</td>
<td>4.25</td>
<td>4.4</td>
<td>103.5</td>
</tr>
<tr>
<td>1:4</td>
<td>2.13</td>
<td>2.2</td>
<td>103.5</td>
</tr>
<tr>
<td>1:8</td>
<td>1.06</td>
<td>1.1</td>
<td>103.5</td>
</tr>
<tr>
<td>1:16</td>
<td>0.53</td>
<td>0.5</td>
<td>94.1</td>
</tr>
</tbody>
</table>
Recovery test. Two plasma samples with low angiotensin I concentration were tested as such and after mixing with increasing amounts of angiotensin I.

<table>
<thead>
<tr>
<th>Added concentration, ng/mL</th>
<th>Expected concentration, ng/mL</th>
<th>Measured concentration, ng/mL</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–</td>
<td>11.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>7.1</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>10.6</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>30.6</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>9.3</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>12.8</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>32.8</td>
<td>30.7</td>
</tr>
</tbody>
</table>

10. LIMITATIONS OF THE PROCEDURE

The clinical significance of the PRA assay is unreliable, if performed on patients who are not kept under controlled conditions of sodium and potassium intake and of posture, or have been administered with drugs such as diuretics, clonidine, beta-blocking agents, estroprogestogens, peripheral vasodilators which affect renin secretion.

Diagnosis should not be established on the basis of a single test result, but should be determined in conjunction with clinical findings and other diagnostic procedures as well as in association with medical judgement.

Bacterial contamination or repeated freeze-thaw cycles of the specimens may affect the assay results.

A skillful technique and strict adherence to the instructions are necessary to obtain reliable results. In particular, precise pipetting and accurate aspiration are essential.

Non-reproducible results may arise from methodological factors, such as:
- cross-exchange of vial caps
- use of the same tip when withdrawing from different vials or dispensing different samples
- leaving the vials open for long
- exposure of reagents or samples to intense heat or heavy sources of bacterial contamination
- inadequate aspiration of incubation mixture
- contamination of tube rims by tracer or samples
- casual oscillations or inadequate handling of the gamma counter
- use of reagents from different master batches.

11. WARNINGS AND PRECAUTIONS

Test components contain sodium azide as a preservative. Because sodium azide may form explosive lead or copper azide in plumbing, it is recommended that drains be thoroughly flushed with water after disposal of solutions containing sodium azide (Council Directive 99/45/EC).

R 22 – Harmful if swallowed.
R 31 – Contact with acids liberates toxic gas.
S 28 – After contact with skin, wash immediately with plenty of water.
S 45 – In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

R 22 – Harmful if swallowed.
R 36/38 – Irritating to eyes and skin.
S 45 – In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

R 11 – Highly flammable.
S 7 – Keep container tightly closed.
S 16 – Keep away from sources of ignition. No smoking.
All serum and plasma units used to produce the components provided in this kit have been tested for the presence of HBsAg, anti-HCV, and anti-HIV-1/2 and found to be non-reactive. As, however, no test method can offer absolute assurance that pathogens are absent, all specimens of human origin should be considered potentially infectious and handled with care.

12. SAFETY PRECAUTIONS
- Do not eat, drink, smoke or apply cosmetics in the assay laboratory.
- Do not pipette solutions by mouth.
- Avoid direct contact with all potentially infectious materials by using protective clothing such as lab coats, protective glasses and disposable gloves. Wash hands thoroughly at the end of each assay.
- Avoid splashing or forming an aerosol. Any reagent spills should be washed with a 5% sodium hypochlorite solution and disposed of as though potentially infectious.
- All samples, biological reagents and materials used in the assay must be considered potentially able to transmit infectious agents. They should therefore be disposed of in accordance with the prevailing regulations and guidelines of the agencies holding jurisdiction over the laboratory, and the regulations of each Country. Disposable materials must be incinerated; liquid waste must be decontaminated with sodium hypochlorite at a final concentration of 5% for at least half an hour. Any materials to be reused must be autoclaved using an overkill approach (USP 24, 2000, p. 2143). A minimum of one hour at 121°C is usually considered adequate, though the users must check the effectiveness of their decontamination cycle by initially validating it and routinely using biological indicators.

13. BASIC RULES OF RADIATION SAFETY
Reagents Containing Iodine-125
This kit contains radioactive material that does not exceed 2.1 µCi (76 kBq) of iodine-125. Appropriate precautions and good laboratory practices should be used in the storage, handling, and disposal of this material.
For practitioners or institutions receiving radioisotopes under a general license:
This radioactive material may be received, acquired, possessed, and used only by physicians, veterinarians in the practice of veterinary medicine, clinical laboratories or hospitals, and only for in vitro clinical or laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations and the general license of the U.S. Nuclear Regulatory Commission or of the state with which the Commission has entered into an agreement for the exercise of regulatory authority.
1. Storage of radioactive material should be limited to a specifically designated area.
2. Access to radioactive materials must be limited to authorized personnel only.
3. Do not pipette radioactive material by mouth.
4. Do not eat or drink within designated radioactive work areas.
5. Areas where spills may occur should be wiped up, then washed with an alkali detergent or radiological decontamination solution. Any glassware used must be rinsed completely with water before washing with other laboratory glassware.

For practitioners or institutions receiving radioisotopes under a specific license:
The receipt, use, transfer and disposal of radioactive materials are subject to the regulations and conditions of your specific license.
ATTENTION: Radioactivity printed in the package insert may be slightly different from the radioactivity printed on the box label and on the tracer vial label. The box label and the tracer vial label indicate the actual amount of radioactivity at the calibration date where the package insert indicates the theoretical radioactivity of the kit.
SCHEME OF THE ASSAY

1 - RECONSTITUTE REAGENTS.

2 - PERFORM ANGIOTENSIN I GENERATION FOR 90 MIN AT 37°C IN NON-COATED TUBES.

3 - IDENTIFY COATED TUBES FOR THE RIA ASSAY IN DUPLICATE.

4 - DISPENSE REAGENTS BY THE FOLLOWING SCHEME AND MIX THE INCUBATION MIXTURE:

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>TUBES</th>
<th>CAL 0-5</th>
<th>SAMPLES &amp; BLANKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CALIBRATORS</td>
<td>50 µL</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SAMPLES</td>
<td>–</td>
<td>50 µL</td>
<td>–</td>
</tr>
<tr>
<td>TRACER</td>
<td>500 µL</td>
<td>500 µL</td>
<td>–</td>
</tr>
</tbody>
</table>

5 - INCUBATE FOR A TIME RANGING FROM 3 TO 24 HOURS AT ROOM TEMPERATURE.

6 - CAREFULLY ASPIRATE THE INCUBATION MIXTURE.

7 - MEASURE THE RADIOACTIVITY OF TUBES.