

Rat Acylated Ghrelin ELISA

Cat. No.: RD394062400R

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Use only the actual version of Product Data Sheet enclosed with the kit!

1. Intended Use

The RD394062400R Rat Acylated Ghrelin ELISA is a sandwich enzyme immunoassay for quantitative measurement of rat acylated ghrelin in EDTA plasma and buffer solution. It is intended for in vitro and research use only.

Features

- The kit measures plasma acylated ghrelin.
- Special blood collection procedure is needed.
- Quality controls are rat-plasma based.

2. Storage, Expiration

Store the kit at -20°C. Under these conditions, the kit is stable until expiration date (see label on the box).

3. Summary

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is synthesized principally in the stomach. It stimulates food intake and transduces signals to hypothalamic regulatory nuclei that controls energy homeostasis. The peptide consists of 28 amino acids, with a n-octanoylation of the serine-3 residue, which is necessary for the biological activity mentioned below. Ghrelin is present in the peripheral circulation under two forms: acylated and unacylated. The Rat Acylated Ghrelin ELISA kit specifically measures the acylated form of ghrelin.

4. Test Principle

The BioVendor Rat Acylated Ghrelin ELISA is based on a double-antibody sandwich technique. The wells of the plate supplied with the kit are coated with a monoclonal antibody specific to the C-terminal part of ghrelin. This antibody will bind to any ghrelin introduced into the wells (standard or sample). The acetylcholinesterase (AChE) - Fab' conjugate which recognizes the N-terminal part of acylated ghrelin is also added to the wells. This allows the two antibodies to form a sandwich by binding on different parts of the rat acylated ghrelin.

The sandwich is immobilized on the plate so the excess reagents may be washed away. The concentration of the rat acylated ghrelin is then determined by measuring the enzymatic activity of the immobilized AChE using the Ellman's Reagent. The AChE tracer acts on the Ellman's Reagent to form a yellow compound. The intensity of the color, which is determined by spectrophotometry, is proportional to the amount of the rat acylated ghrelin present in the well during the immunological incubation.

5. Precautions

- For *in vitro* research use only.
- Avoid contact with the Substrate Solution (Ellman's Reagent). In the case of contact, wash your skin thoroughly with water.
- Wear gloves and laboratory coats when handling immunodiagnostic materials.
- The materials should not be pipetted by mouth.
- Do not drink, eat or smoke in rooms where immunodiagnostic materials are being handled.
- Do not mix different lot numbers of any kit components.

6. Reagents Supplied

| <i>Kit Components</i> | <i>Quantity</i> |
|--|-----------------|
| Antibody Coated Microtiter Strips, ready to use | 96 wells |
| Conjugate Solution, lyophilized | 1 vial |
| Rat Acylated Ghrelin Standard, lyophilized | 2 vials |
| Quality Control, lyophilized | 2 vials |
| Dilution Buffer, lyophilized | 1 vial |
| Wash Solution Concentrate, liquid | 1 vial |
| Tween 20, liquid | 1 vial |
| Substrate Solution (Ellman's reagent), lyophilized | 2 vials |
| Product Data Sheet + Certificate of Analysis | 1 pc |
| Template Sheet + Cover Sheet | 1 pc |

Each kit contains sufficient reagents for 96 wells. The kit can be used in two independent runs.

7. Materials Required but Not Supplied

- Potassium Phosphate buffer 0.1 M pH 7.4
- NaOH 10M
- p-hydroxymercuribenzoic acid (PHMB, *e.g. FLUKA Cat. No.: 55540 or another reagent of equivalent quality*)
- HCl 1M
- EDTA-plasma collection test tubes
- Test tubes for diluting samples (polypropylene)
- Precision pipettes to deliver 20-1000 μ l and disposable tips
- Multichannel pipette 100-200 μ l (recommended)
- Microplate reader with 405 or 414 nm filter
- Orbital microplate shaker
- Software package facilitating data generation and analysis
- Microtitration plate washer (optional) [Manual washing is possible but not preferable.]
- Glassware (graduated cylinder and bottle for Wash Solution)
- Deionized (distilled) water

8. Preparation of Reagents

Dilution Buffer:

Reconstitute one vial with 50 ml of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C is 1 month.

Rat Acylated Ghrelin Standard:

Reconstitute the vial with 1 ml of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the acylated ghrelin standard S1 is 250 pg/ml.

Use seven polypropylene tubes to prepare the set of standards S2 to S8 from the reconstituted lyophilized standard S1 by serial dilution with Dilution Buffer as follows:

| <i>Standard to be prepared</i> | <i>Dilution buffer volume</i> | <i>Standard to be added</i> |
|--------------------------------|-------------------------------|-----------------------------|
| S1 (250 pg/ml) | ----- | ----- |
| S2 (125 pg/ml) | 500 µl | S1: 500 µl |
| S3 (62.5 pg/ml) | 500 µl | S2: 500 µl |
| S4 (31.3 pg/ml) | 500 µl | S3: 500 µl |
| S5 (15.6 pg/ml) | 500 µl | S4: 500 µl |
| S6 (7.81 pg/ml) | 500 µl | S5: 500 µl |
| S7 (3.91 pg/ml) | 500 µl | S6: 500 µl |
| S8 (1.96 pg/ml) | 500 µl | S7: 500 µl |

Stability at -20°C is 1 week.

Quality Control:

Reconstitute the vial with 1 ml of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at -20°C is 1 week.

Conjugate Solution (Anti-acylated ghrelin-AChE tracer):

Reconstitute one vial with 10 ml of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C is 1 week.

Wash Solution:

Dilute 1 ml of Wash Solution Concentrate to 400 ml with distilled or deionized water. Add 200 µl of Tween 20 (use a magnetic stirrer to mix the contents).

Stability at 4°C is 1 week.

Substrate Solution (Ellman's Reagent):

Reconstitute one vial with 49 ml of distilled or deionized water and 1 ml of Wash Solution Concentrate five minutes before use. The tube contents should be thoroughly mixed. Stability at 4°C and in the dark is 1 day.

9. Preparation of Samples

Precautions!

All samples must be free of organic solvents prior to assay.

Samples should be assayed immediately after collection or should be stored at -20°C.

Blood collection:

Blood samples are collected in tubes containing EDTA and p-hydroxymercuribenzoic acid (1 mM in the final sample volume) to prevent the degradation of acylated ghrelin by protease. Samples are centrifuged at 3,500 rpm for 10 min at +4°C and then, supernatants are transferred in separate tubes. Add immediately 100 µl of 1M HCl per ml of collected plasma and centrifuge them at 3,500 rpm for 5 min at +4°C. Then, supernatants are transferred in separate tubes. Samples should be quickly assayed or stored at -20°C for later use. For the preparation of PHMB, we suggest preparing a 100 times concentrate solution (100 mM) in potassium phosphate buffer containing 1.2% NaOH 10M (v/v) and then, adding 10 µl of this solution per ml of blood.

For assaying the unacylated ghrelin, please refer to the section "Blood collection" of the protocol of the RD394063400 Rat Unacylated Ghrelin ELISA.

Sample preparation:

Plasma samples may be directly assayed (without any extraction procedure) after being diluted at least to 1:5 in the Dilution Buffer in order to avoid matrix effect.

10. Assay Procedure

All reagents and samples need to reach room temperature prior to the assay

- 1) Select strips sufficient for your assay and wash the wells 5-times with the prepared Wash Solution (0.3 ml per well) just before further using the strips.
Place the unused strips back in the packet and store at 4°C.
- 2) Left four wells empty for blanking the Substrate Solution (BI).
- 3) Pipette 100 µl of prepared Dilution buffer into four Non Specific Binding wells (NSB).
- 4) Pipette 100 µl of each of the eight prepared standards (S1 to S8) and Quality Control, preferably in duplicates into the respective wells.
- 5) Pipette 100 µl of diluted samples into the appropriate wells, preferably in duplicates (see example of work sheet).
- 6) Pipette 100 µl of Conjugate Solution to each well, except the Blank (BI) wells.
- 7) Cover the plate with adhesive sheet.
- 8) Incubate the plate 3 hours at room temperature (ca. 25°C) or 20 hours at 4°C.
The long incubation period allows the increase of the assay sensitivity: 0.2 pg/ml versus 0.7 pg/ml for short incubation.
- 9) Reconstitute the Substrate Solution.
- 10) Wash the wells 5-times with the Wash Solution (0.3 ml per well). Do not empty the wells after the fifth washing step, but shake the plate slightly for 5 minutes using an orbital shaker.
- 11) Repeat washing the wells 5-times with the wash buffer (0.3 ml per well).
- 12) Add 200 µl of Substrate Solution into each well including the blank (BI) wells.
- 13) Incubate the plate in darkness at room temperature (using an orbital shaker is optimal for the color development).
- 14) Determine the absorbance by reading the plate at 405 to 414 nm: 30 minutes after adding the Substrate Solution for long first incubation period (20 hours at +4°C) or 30 to 60 minutes after adding the Substrate Solution for short first incubation period (3 hours at room temperature).

Example of work sheet:

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-----|----|----|----|----|----|----|----|----|----|----|----|
| A | BI | S1 | S1 | QC | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |
| B | BI | S2 | S2 | QC | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |
| C | BI | S3 | S3 | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |
| D | BI | S4 | S4 | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |
| E | NSB | S5 | S5 | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |
| F | NSB | S6 | S6 | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |
| G | NSB | S7 | S7 | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |
| H | NSB | S8 | S8 | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa | Sa |

11. Calculations

Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of standards versus *log* of the known concentration (X) of standards, using the four-parameter function. The results are reported as the acylated ghrelin concentration in samples (pg/ml). Make sure that your plate reader has subtracted the absorbance readings of the blank wells from absorbance readings of the rest of the plate. If not, do it manually.

Alternatively, the *logit log* function can be used to linearize the calibration curve (i.e. *logit* of absorbance (Y) is plotted versus *log* of the known concentration (X) of standards).

The actual concentration of acylated ghrelin in the original plasma sample has been assessed by multiplying the assay result by the dilution factor (due notably to the minimal dilution for the assay 1:5 and the addition of 1M HCl).

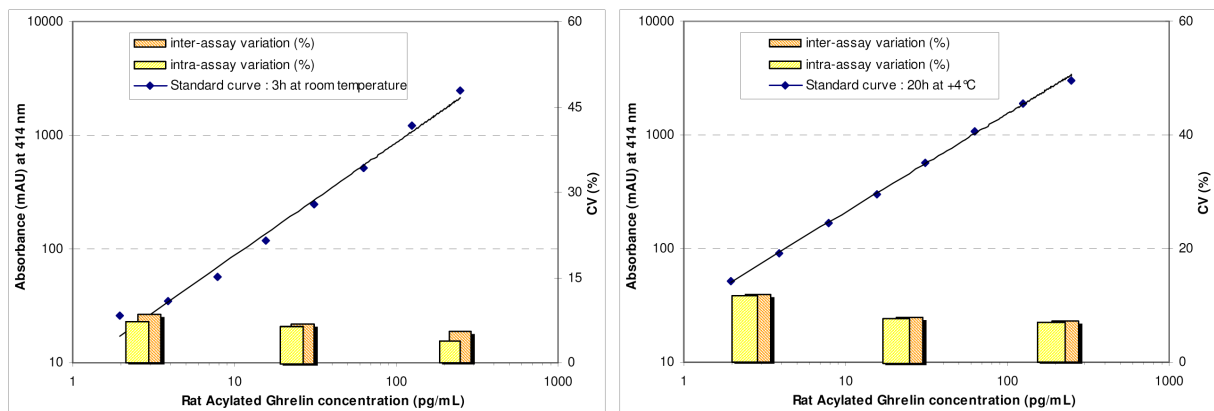
12. Limits of Assay

Results exceeding concentration 250 pg/ml should be repeated with more diluted samples (e.g. 1:10). In this case, dilution factor need to be taken into consideration in calculating of the concentrations.

Non Specific Binding Absorbance <0.050 is acceptable.

13. Performance Characteristics

Typical analytical data of BioVendor Rat Acylated Ghrelin ELISA are presented in this chapter. The following data are for demonstration purpose only. Your data may be different and still correct. These data were obtained using all reagents as supplied in this kit under the following conditions: 30 minutes developing at room temperature for long first incubation period (20h at +4°C) and 60 minutes developing for short first incubation period (3h at RT), reading at 414 nm. A 4-parameter logistic fitting was used to determine the concentrations.



Example data:

| Ghrelin standard (pg/ml) | Absorbances | |
|--------------------------|---------------------------------------|---|
| | Short first incubation period (3h RT) | Long first incubation period (20h +4°C) |
| 250 | 2.457 | 3.007 |
| 125 | 1.214 | 1.897 |
| 62.5 | 0.514 | 1.084 |
| 31.3 | 0.247 | 0.571 |
| 15.6 | 0.118 | 0.304 |
| 7.81 | 0.057 | 0.166 |
| 3.91 | 0.035 | 0.090 |
| 1.95 | 0.026 | 0.051 |

- **Sensitivity**

The Rat Acylated Ghrelin ELISA has been validated for its use in buffer and in plasma (without extraction but diluted at least 1:5). A sigmoidal 4-parameter logistic fitting was used to determine the concentrations.

The Limit of Determination, calculated as the concentration of acylated ghrelin corresponding to the NSB average (n = 8) plus three standard deviations is 0.2 pg/ml and 0.7 pg/ml for long and short first incubation period, respectively.

The Limit of Quantification. Due to the minimal plasma dilution needed (1:5), the limits of quantification in the samples are 1 pg/ml (20h at +4°C) and 3.5 pg/ml (3h at RT), respectively.

- **Precision**

Intra-assay (Within-Run), Inter-assay (Run-to-Run) and Spiking Recovery

The intra-assay and inter-assay variations were studied on 30 rat plasma (free of ghrelin) spiked samples for each level of QC. QCs were prepared as 5x concentrated from a pool of rat plasma and then diluted to 1:5 in Dilution Buffer before assay. Replicate samples (n=6) at each of the three validation levels were analysed along with the calibration curve for a total of 5 independent runs.

| <i>Samples</i> | <i>Expected concentrations in diluted QC (pg/ml)</i> | <i>Observed concentrations (pg/ml)</i> | <i>Intra-assay CV (%)</i> | <i>Inter-assay CV (%)</i> | <i>Recovery O/E (%)</i> | <i>Confidence Interval ($\alpha = 0.05$)</i> |
|---|--|--|---------------------------|---------------------------|-------------------------|---|
| Incubation 20 hours at +4°C | | | | | | |
| QC1 | 2 | 1.91 | 11.2 | 11.4 | 95.4 | 95.4 ± 4.4 |
| QC2 | 25 | 24.9 | 6.8 | 7.0 | 99.5 | 99.5 ± 2.9 |
| QC3 | 200 | 203 | 6.7 | 7.0 | 102 | 102 ± 3.1 |
| Incubation 3 hours at room temperature | | | | | | |
| QC1 | 2 | 2.12 | 7.0 | 8.1 | 106 | 106 ± 4.5 |
| QC2 | 25 | 24.7 | 5.7 | 6.1 | 98.6 | 98.6 ± 2.8 |
| QC3 | 200 | 190 | 3.7 | 5.4 | 94.8 | 94.8 ± 3.4 |

- **Linearity**

Rat plasma samples were diluted 1:5. Afterwards, four independent dilutions (n=3) were performed and the samples were assayed.

| <i>Samples</i> | <i>Dilution factor</i> | <i>Acylated ghrelin measured (pg/ml)</i> | <i>Corrected Concentrations (pg/ml)</i> | <i>Recovery (%)</i> | <i>Mean Recovery (%)</i> |
|----------------|------------------------|--|---|---------------------|--------------------------|
| 1 | 1:5 | 26.3 | 132 | - | 109 |
| | 1:10 | 13.9 | 139 | 105 | |
| | 1:20 | 7.20 | 144 | 109 | |
| | 1:25 | 5.68 | 142 | 108 | |
| | 1:50 | 3.03 | 152 | 115 | |
| 2 | 1:5 | 48.2 | 241 | - | 105 |
| | 1:10 | 24.4 | 244 | 101 | |
| | 1:20 | 12.6 | 252 | 105 | |
| | 1:25 | 10.2 | 255 | 106 | |
| | 1:50 | 5.18 | 259 | 107 | |
| 3 | 1:5 | 48.7 | 244 | - | 99.8 |
| | 1:10 | 25.8 | 258 | 106 | |
| | 1:20 | 12.9 | 258 | 106 | |
| | 1:25 | 9.54 | 239 | 98.0 | |
| | 1:50 | 4.36 | 218 | 89.3 | |

- **Effect of Sample Matrix**

Five individual lots of human plasma samples were tested. Validation samples (n=3) were prepared five times concentrated in each matrix (free of ghrelin) and then diluted to 1:5 in order to obtain a final concentration of 25 pg/ml. Sample concentrations were read from a calibration curve derived from a pool of rat plasmas.

| <i>Sample</i> | <i>Expected (pg/ml)</i> | <i>Observed (pg/ml)</i> | <i>Recovery (%)</i> | <i>Mean Recovery (%)</i> |
|---------------|-------------------------|-------------------------|---------------------|--------------------------|
| 1 | 25 | 24.6 | 98.4 | 97.7 |
| 2 | | 23.2 | 92.8 | |
| 3 | | 24.0 | 96.0 | |
| 4 | | 24.8 | 99.2 | |
| 5 | | 25.4 | 102 | |

- **Effect of Freezing/Thawing (sample stability)**

Four human plasma samples (n=3) were analyzed just after collection and dilution to 1:5 before the assay (expected value) and after 1, 2 and 3 freeze/thaw cycles.

| Samples | Expected value (pg/ml) | Observed 1 cycle (pg/ml) | Observed 2 cycles (pg/ml) | Observed 3 cycles (pg/ml) | Mean recovery O/E (%) |
|---------|---------------------------|--------------------------------|---------------------------------|---------------------------------|-----------------------------|
| 1 | 140 | 129 | 108 | 102 | 80.7 |
| 2 | 268 | 263 | 225 | 227 | 88.9 |
| 3 | 192 | 183 | 151 | 152 | 84.4 |
| 4 | 221 | 266 | 222 | 228 | 108 |

- **Specificity**

The Rat Acylated Ghrelin ELISA is highly specific. The cross-reactivity values for related peptides have been as follows:

Cross-reactivity:

| | |
|--|----------|
| - Ghrelin (Human): | 82 % |
| - Ghrelin (Des-Octanoyl-Ser ³) (Rat, Mouse): | <0.001 % |
| - Ghrelin (Des-Octanoyl-Ser ³) (Human): | <0.001 % |
| - Ghrelin (1-11) (Rat): | <0.001 % |
| - Ghrelin (1-14) (Human): | <0.001 % |
| - Ghrelin (17-28) (Human, Rat): | <0.001 % |
| - GHRF (rat): | <0.001 % |
| - Insulin (Rat): | <0.001 % |
| - Motiline: | <0.001 % |
| - Leptin (Rat): | <0.001 % |
| - Somatostatine: | <0.001 % |
| - CRF (Human, Rat): | <0.001 % |
| - Glucagon (Human, Rat): | <0.001 % |

14. Troubleshooting and FAQs

1/ Weak signal in all wells

Possible explanations:

- Omission of a reagent or a step
- Improper preparation or storage of a reagent
- Assay performed before reagents were allowed to come to room temperature
- Incubation in wrong conditions (time or temperature) or reading time too short

2/ High signal and background (NSB) in the wells

Possible explanations:

- Improper or inadequate washing
- Overdeveloping; substrate incubation time should be decreased
- Contamination of NSB wells with standard or sample

3/ High coefficient of variation (CV)

Possible explanation:

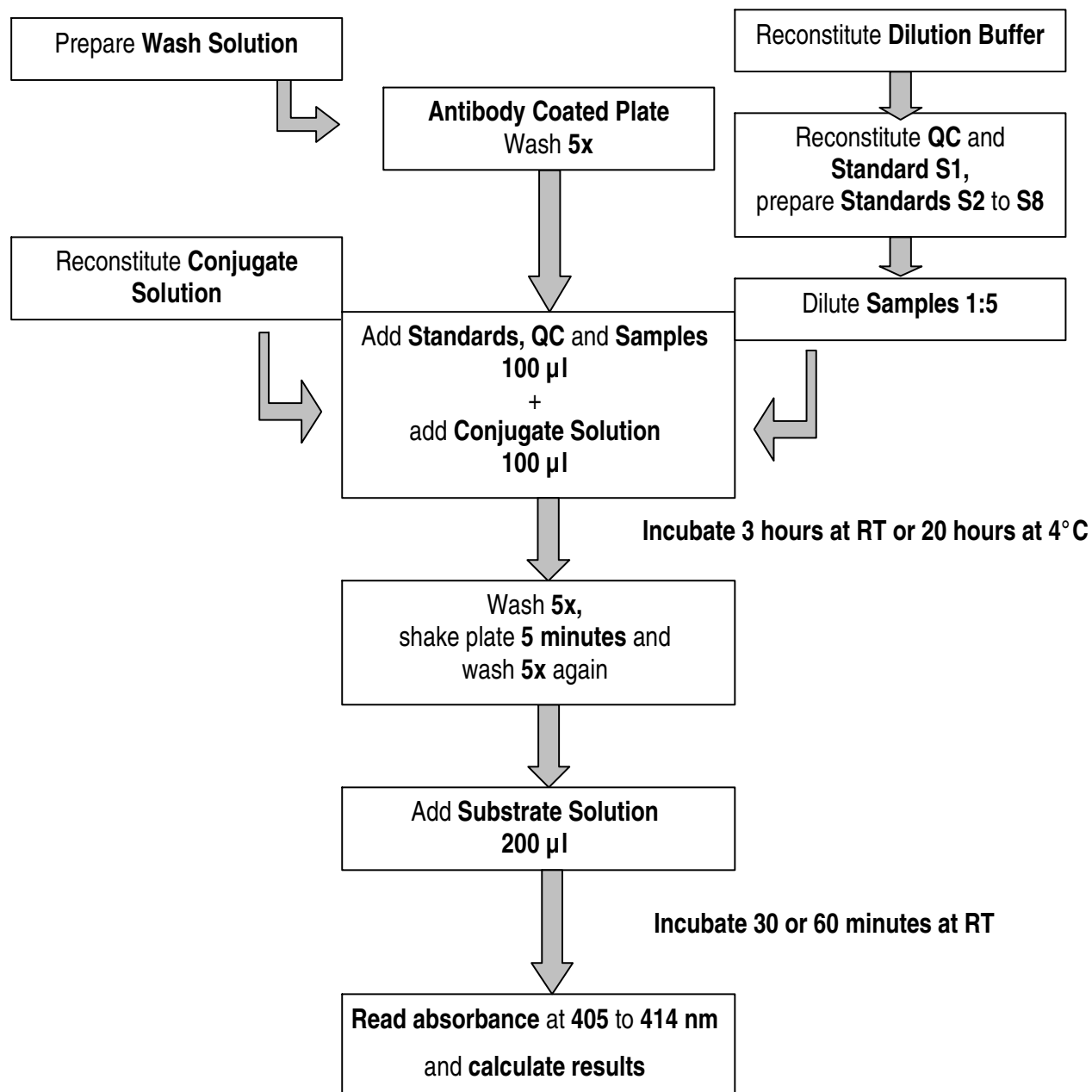
- Improper or inadequate washing
- Poor pipetting technique

15. References

Grassi J. & Pradelles Ph. Compounds labelled by the acetylcholinesterase of *Electrophorus Electricus*. Its preparation process and its use as a tracer or marker in enzyme-immunological determinations. *United States patent, N° 1,047,330. September 10, 1991*

Notes:

Assay Procedure Summary



The BioVendor RD394062400R Rat Acylated Ghrelin ELISA is manufactured and distributed on agreement of the Société de Pharmacologie et d'Immunologie - BIO, Parc d'activités du Pas du Lac 10 bis, Avenue Ampère, F-78180 Montigny le Bretonneux, FRANCE



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