Human Leptin ELISA

NB-06-0537

1. INTENDED USE

The Human Leptin ELISA, is a sandwich enzyme immunoassay for the quantitative measurement of human leptin.

FOR RESEARCH USE ONLY.

Features

- The total assay time is less than 2.5 hours.
- The kit measures total leptin in serum and plasma (EDTA, citrate, heparin) and tissue culture medium.
- Assay format is 96 wells.
- Quality Controls are human serum based. No animal sera are used.
- Standards are recombinant protein based.
- Components of the kit are provided ready to use, concentrated or lyophilized.

2. STORAGE, EXPIRATION

Store the complete kit at 2-8°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

3. TEST PRINCIPLE

In the Neo Biotech Human Leptin ELISA, Standards, Quality Controls and samples are incubated in microplate wells pre-coated with polyclonal anti-human leptin antibody. After 60 minutes incubation and washing, polyclonal anti-human leptin antibody, conjugated with horseradish peroxidase (HRP) is added to the wells and incubated for 60 minutes with captured leptin. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to the concentration of leptin. A standard curve is constructed by plotting absorbance values against concentrations of Standards, and concentrations of unknown samples are determined using this standard curve.

4. PRECAUTIONS

- **For research use only**.
- Wear gloves and laboratory coats when handling biological materials.
- Do not drink, eat or smoke in the areas where biological materials are being handled.
- This kit contains components of human origin. These materials were found non-reactive for HBsAg, HCV antibody and for HIV 1/2 antigen and antibody. However, these materials should be handled as potentially infectious, as no test can guarantee the complete absence of infectious agents.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents. Stop and/or Substrate Solutions may cause skin/eyes irritation. In case of contact with the Stop Solution and the Substrate Solution wash skin/eyes thoroughly with water and seek medical attention, when necessary.
- The materials must not be pipetted by mouth.

5. TECHNICAL HINTS

- Reagents with different lot numbers should not be mixed.
- Use thoroughly clean glassware.
- Use deionized (distilled) water, stored in clean containers.
- Avoid any contamination among samples and reagents. For this purpose, disposable tips should be used for each sample and reagent.
- Substrate Solution should remain colourless until added to the plate. Keep Substrate Solution protected from light.
- Stop Solution should remain colourless until added to the plate. The colour developed in the wells will turn from blue to yellow immediately after the addition of the Stop Solution. Wells that are green in colour indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.
- Dispose of consumable materials and unused contents in accordance with applicable national regulatory requirements.

6. REAGENT SUPPLIED

Kit Components	*State*	*Quantity*
Antibody Coated Microtiter Strips	ready to use	96 wells
Conjugate Solution	ready to use	13 ml
Master Standard	lyophilized	1 vial
Quality Control High	lyophilized	1 vial
Quality Control Low	lyophilized	1 vial
Dilution Buffer	ready to use	13 ml
Wash Solution Concentrate (10x)	concentrated	100 ml
Substrate (TMB) Solution	ready to use	13 ml
Stop Solution	ready to use	13 ml
Product Data Sheet + Certificate of Analysis		1 pc

7. MATERIAL REQUIRED BUT NOT SUPPLIED

- Deionized (distilled) water
- Test tubes for diluting samples
- Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
- Precision pipettes to deliver 10-1000 µl with disposable tips
- Multichannel pipette to deliver 100 µl with disposable tips
- Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
- Vortex mixer
- Orbital microplate shaker capable of approximately 300 rpm
- Microplate washer (optional). [Manual washing is possible but not preferable.]
- Microplate reader with 450 ± 10 nm filter, preferably with reference wavelength 630 nm (alternatively another one from the interval 550-650 nm)
- Software package facilitating data generation and analysis (optional)

8. PREPARATION OF REAGENTS

All reagents need to be brought to room temperature prior to use. Always prepare only the appropriate quantity of reagents for your test. Do not use components after the expiration date marked on their label.

- **Assay reagents supplied ready to use:**

Antibody Coated Microtiter Strips

Stability and storage:

Return the unused strips to the provided aluminum zip-sealed bag with desiccant and seal carefully. Remaining Microtiter Strips are stable 3 months stored at 2-8C and protected from the moisture**.**

Conjugate solution
Dilution Buffer
Substrate Solution
Stop Solution

Opened reagents are stable 3 months when stored at 2-8C

- **Assay reagents supplied concentrated or lyophilized:**

Human Leptin Standards

Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution of Set of Standards!

Reconstitute the lyophilized Set of Standards with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam). The resulting concentration of the leptin in the stock solutions is 50ng/ml

Prepare sets of standards using Dilution Buffer as follows:

Volume of Standard	*Dilution Buffer*	*Concentration*
Stock	-	50 ng/ml
200 µl of stock	300 µl	20 ng/ml
250 µl of 20mg/ml	250 µl	10 ng/ml
250 µl of 10ng/ml	250 µl	5 ng/ml
200 µl of 5ng/ml	300 µl	2 ng/ml
250 µl of 2 ng/ml	250 µl	1 ng/ml

The volume of dilution buffer for reconstitution of Master standard given in CoA dilutes standards 3x, the same is samples and controls.

Prepared Set of Standards are ready to use, do not dilute them.

Stability and storage:

The reconstituted Set of Standards have to be used immediately or to be stored frozen at -20C for 3 months. Avoid repeated freeze/thaw cycles.

Quality Controls High, Low

Refer to the Certificate of Analysis for current Quality Controls concentrations!

Reconstitute each Quality Control (High and Low) with 350 ul of distilled water just prior to the assay. Let it dissolve at least 30 minutes with occasional gentle shaking (not to foam).

Dilute reconstituted Quality Controls 3x with Dilution Buffer, e.g. 50 ul Quality Control + 100 ul Dilution Buffer when assaying samples in singlets, or preferably 100 ul Quality Control + 200 ul Dilution Buffer for duplicates.

Stability and storage:

The reconstituted Quality Controls must be used immediately or aliquoted and frozen at -20°C for 3 month. Avoid repeated freeze/thaw cycles.

Do not store the diluted Quality Controls.

Wash Solution Concentrate (10x)

Dilute Wash Solution Concentrate (10x) ten-fold in distilled water to prepare a 1x working solution. Example: 100 ml of Wash Solution Concentrate (10x)+ 900 ml of distilled water for use of all 96- wells.

Stability and storage:

The diluted Wash Solution is stable 1 month when stored at 2-8°C. Opened Wash Solution Concentrate (10x) is stable 3 months when stored at 2-8°C.

The kit measures leptin in serum or plasma. (EDTA,citrate,heparin)

Samples should be assayed immediately after collection or should be stored at -20°C. Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

Dilute samples 3x with Dilution Buffer just prior to the assay (e.g. 50 ul of sample + 100 ul of Dilution Buffer for singlets, or preferably 100 ul of sample + 200 ul of Dilution Buffer for duplicates). **Mix well** (not to foam). Vortex is recommended.

Samples should be stored at -20°C, or preferably at -70°C for long-term storage. Avoid repeated freeze/ thaw cycles.

Do not store the diluted samples.

See Chapter 13 for stability of serum and plasma samples if stored at 2-8°C, effect of freezing/thawing and effect of sample matrix (serum/plasma) on the concentration of leptin.

Note: It is recommended to use a precision pipette and a careful technique to perform the dilution in order to get precise results.

Ask for protocol at info@neo-biotech.com if assaying tissue culture medium.

9. ASSAY PROCEDURE

1. Pipet **100 ul** of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells.
2. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
3. Wash the wells **3-times** with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add **100 ul** of Conjugate Solution into each well.
5. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
6. Wash the wells **3-times** with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
7. Add **100 ul** of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
8. Incubate the plate for **10 minutes** at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.
9. Stop the colour development by adding **100 ul** of Stop Solution.
10. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550-650 nm). Subtract readings at 630 nm (550-650 nm) from the readings at 450 nm. **The absorbance should be read within 5 minutes following step 9.**

Note: If some samples and standard/s have absorbances above the upper limit of your microplate reader, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine leptin concentration of off-scale standards and samples. The readings at 405 nm should not replace the readings for samples that were "in range" at 450 nm.

Note 2: Manual washing: Aspirate wells and pepet 0.35 ml Wash Solution into each well. Aspirate wells and repeat twice. After final wash, invert and tap the plate strongly against paper towel. Make certain that Wash Solution has been removed entirely.

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	**Standard 50**	Blank	Sample 8	Sample 16	Sample 24	Sample 32
B	**Standard 20**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	**Standard 10**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	**Standard 5**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	**Standard 2**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	**Standard 1**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	**QC High**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	**QC Low**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

Figure 1: Example of a work sheet.

12. CALCULATIONS

Most microtiter readers perform automatic calculations of analyte concentration. The standard curve is constructed by plotting the mean absorbance (Y) of Standards against the known concentration (X) of Standards in logarithmic scale, using the four-parameter algorithm. Results are reported as concentration of leptin ng/ml in samples.

Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of the mean absorbance (Y) is plotted against log of the known concentration (X) of Standards.

Samples, Quality Controls and Standards are all diluted 3x prior to analysis, so there is no need to take this dilution factor into account.

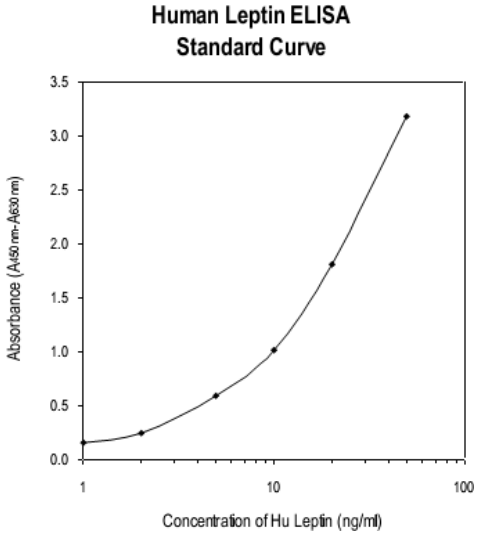

Figure 2: Typical Standard Curve for Human Leptin ELISA

13. PERFORMANCE CHARACTERISTICS

Typical analytical data of Neo Biotech Human Leptin ELISA, are presented in this chapter.

- **Sensitivity**

Limit of Detection (LOD) (defined as concentration of analyte giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: Ablank + 3xSDblank) is calculated from the real leptin values in wells and is 0.2 ng/ml.
*Dilution Buffer is pipetted into blank wells.

- **Limit of assay**

Results exceeding leptin level of 50 ng/ml should be repeated with more diluted samples. Dilution factor needs to be taken into consideration in calculating the leptin concentration.

- **Specificity**

The antibodies used in this ELISA are specific for human leptin

Sera of several mammalian species were measured in the assay. See results below. For details please contact us at info@neo-biotech.com.

Mammalian serum sample	*Observed crossreactivity*
Bovine	No
Goat	No
Hamster	No
Horse	No
Monkey	No
Mouse	No
Pig	No
Rabbit	No
Rat	No
Sheep	No
Cat	No
Dog	No

- **Precision**

Intra-assay (Within-Run) (n=8)

Sample	*Mean (ng/ml)*	*SD (ng/ml)*	*CV (%)*
1	15.01	0.06	4.2
2	3.56	0.02	7.6

Inter assay (Run-to-Run) (n=6)

Sample	*Mean (ng/ml)*	*SD (ng/ml)*	*CV (%)*
1	15.39	1.04	6.7
2	29.34	1.28	4.4

- **Spiking Recovery**

Serum samples were spiked with different amounts of human leptin and assayed.

Sample	***O**bserved (ng/ml)*	***E**xpected (ng/ml)*	*Recovery **O/E** (%)*
1	4.22	-	-
	7.52	8.40	89.5
	11.85	13.37	88.6
	17.41	17.76	98.0
2	14.09	-	-
	17.78	18.27	97.3
	19.92	23.24	85.7
	25.91	27.63	93.8

- **Linearity**

Serum samples were serially diluted with Dilution Buffer and assayed.

Sample	*Dilution*	***O**bserved (ng/ml)*	***E**xpected (ng/ml)*	*Recovery **O/E** (%)*
1	-	20.99	-	-
	2x	10.34	10.49	98.5
	4x	5.32	5.25	101.4
	8x	2.45	2.62	93.4
2	-	29.94	-	-
	2x	15.72	14.97	105.0
	4x	7.80	7.49	104.2
	8x	3.83	3.74	102.3

- **Effect of sample matrix**

Citrate, heparin and EDTA plasmas were compared to respective serum samples from the same 9

individuals. Results are shown below:

Specimen No.	Serum *(ng/)ml)*	Plasma *(ng/ml)*		
		Heparin	Citrate	EDTA
1	7.72	7.41	6.47	7.13
2	9.05	7.84	6.57	8.95
3	2.54	2.18	1.81	2.32
4	7.08	6.13	5.97	7.47
5	18.71	16.94	13.81	17.55
6	19.64	16.01	15.05	23.39
7	6.42	6.31	5.65	6.76
8	3.97	3.93	3.32	3.36
9	5.67	7.17	6.38	5.84
Mean (ng/ml)	**8.97**	**8.21**	**7.22**	**9.19**
Mean Plasma/Serum (%)	**-**	**91.6**	**80.6**	**102.7**
Correlation. coeff. R^2	**-**	**0.97**	**0.97**	**0.96**

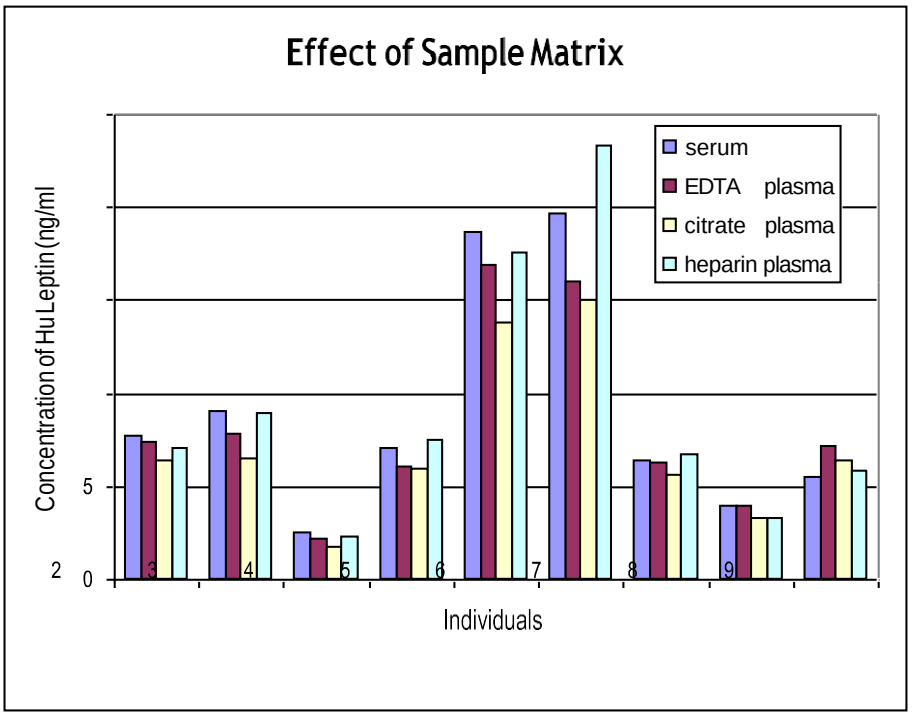

Figure 3: Leptin levels measured using Human Leptin ELISA using serum, EDTA, citrate and heparin plasma, respectively.

- **Stability of samples stored at 2-8°C**

Samples should be stored at –20°C. However, no decline in concentration of leptin was observed in serum and plasma samples after 7 days when stored at 2-8°C. To avoid microbial contamination, samples were treated with ε-aminocaproic acid and sodium azide, resulting in the final concentration

of 0.03% and 0.1%, respectively.

Sample	Incubation Temp, Period	Serum *(ng/ml)*	Plasma *(ng/ml)*		
			EDTA	Citrate	Heparin
1	-20°C	2.03	2.21	1.79	1.90
	2-8°C, 1 day	2.18	2.21	1.83	1.00
	2-8°C, 7 days	2.26	2.21	1.64	2.28
2	-20°C	3.51	3.54	3.26	3..56
	2-8°C, 1 day	3.65	3.79	3.42	2.95
	2-8°C, 7 days	3.74	3.49	3.19	4.09
2	-20°C	8.76	9.20	7.13	8.94
	2-8°C, 1 day	7.80	8.99	8.19	8.56
	2-8°C, 7 days	7.70	8.03	7.87	8.43

- **Effect of Freezing/Thawing**

No decline was observed in concentration of human leptin in serum and plasma samples after repeated (5x) freeze/thaw cycles. However, it is recommended to avoid unnecessary repeated freezing/thawing of the samples.

Sample	Number of f/t cycles	Serum *(ng/ml)*	Plasma *(ng/ml)*		
			EDTA	Citrate	Heparin
1	1x	5.90	6.05	5.23	5.63
	3x	5.78	5.49	5.40	5.39
	5x	5.64	5.99	5.47	6.14
2	1x	3.09	3.29	2.85	2.86
	3x	3.21	3.28	2.81	2.71
	5x	3.44	3.41	2.72	3.54
3	1x	4.63	5.33	4.71	4.80
	3x	3.73	4.96	4.67	4.61
	5x	4.58	5.39	4.80	4.91

- **Reference range**

It is recommended that each laboratory include its own panel of control sample in the assay. Each laboratory should establish its own normal and pathological reference ranges for leptin levels with the assay.

14. DEFINITION OF THE STANDARD

A recombinant protein is used as the standard. The recombinant human leptin is a 16 kDa protein containing 147 amino acid residues.

15. METHOD COMPARISON

The Neo Biotech Human Leptin ELISA, was compared to a commercial RIA. Linear regression analysis of the results yielded the following results.

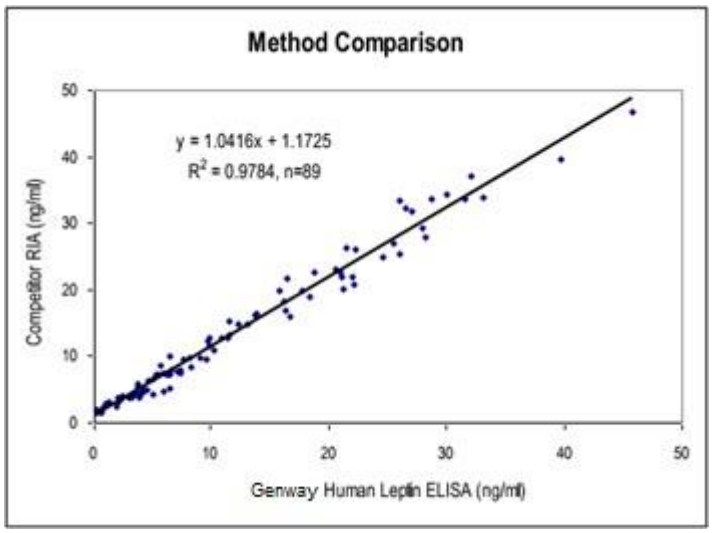

Figure 4: Method comparison

16. TROUBLESHOOTING AND FAQS

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
- Improper wavelength when reading absorbance

High signal and background in all wells

Possible explanations:

- Improper or inadequate washing
- Overdeveloping; incubation time with Substrate Solution should be decreased before addition of Stop Solution
- Incubation temperature over 30°C

High coefficient of variation (CV)

Possible explanation:

- Improper or inadequate washing
- Improper mixing Standards, Quality Controls or samples

17. REFERENCES

References to leptin:

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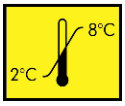
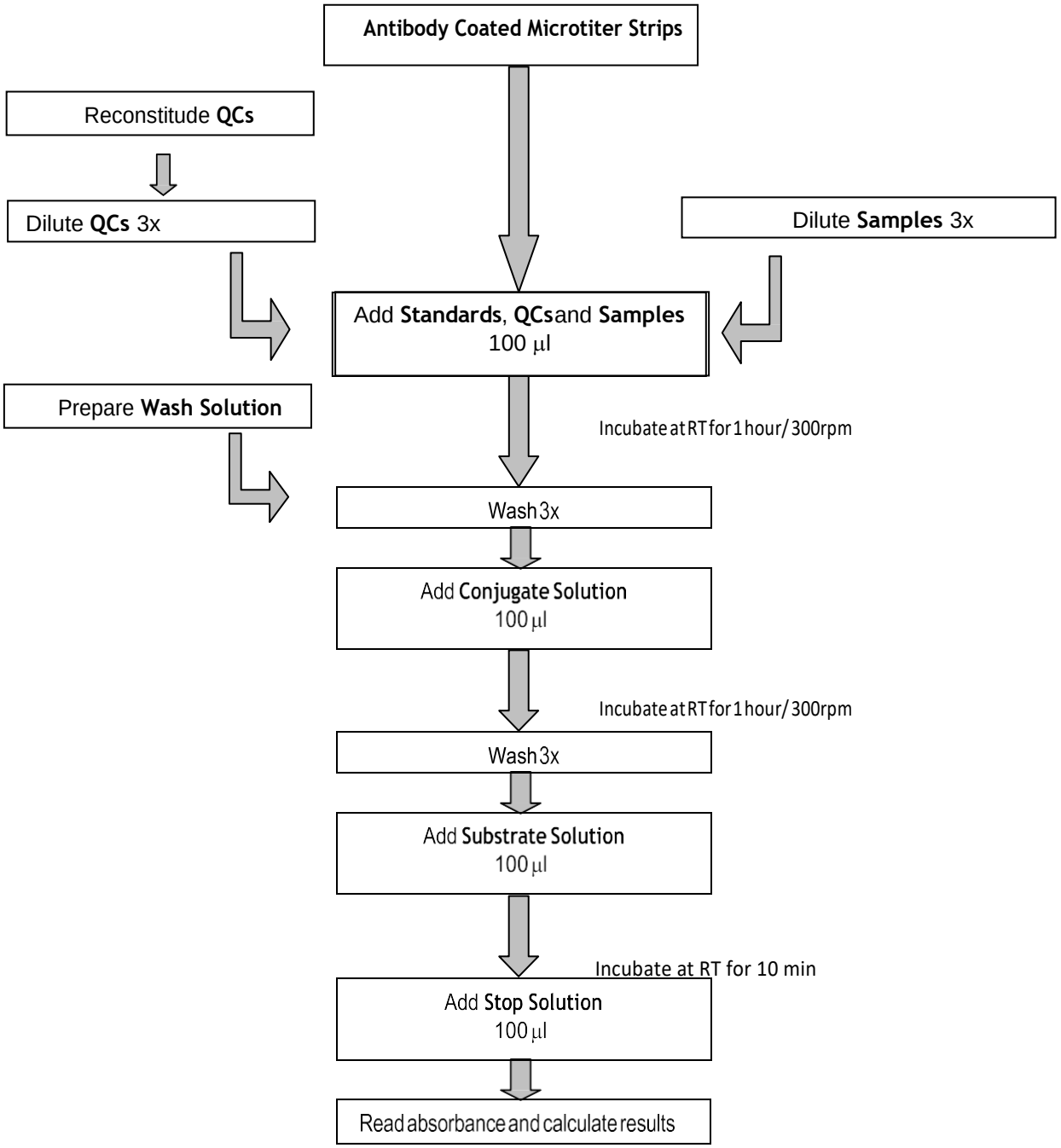
EXPLANATION OF SYMBOLS

REF	Catalogue number
Cont.	Content
LOT	Lot number
	See instructions for use
	Biological hazard
	Expiry date
	Storage conditions
	Identification of packaging materials

Assay Procedure Summary



	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												