BioVendor Group

ENG

Instructions for use:

sHLA-G ELISA

Catalogue number:

RD194070100R

For research use only!

BioVendor R&D

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CONTENT

HISTORY OF CHANGES

Previous version		Current Version
ENG.005.A		ENG.006.A
”History of changes” added.		
Chapter 9.	A sentence “Centrifuge liquid containing microtube vials before opening“ added.	
Chapter 10.	Two diluents are available in the kit. Dilution Buffer 1 corresponds to the original version of the Dilution Buffer (the sole diluent present in the kit up to lot E13-034 / August 2013) and it is recommended for preparation of amniotic fluid samples. Additionally, Dilution Buffer 2 has been included starting from lot E13-055 (September 2013). This new diluent enables users to dilute EDTA plasma 8-fold and, thus, to further suppress potential matrix effects. Consequently, higher signal and better analytical characteristics are reached for EDTA plasma samples when using this new buffer.	Two diluents are available in the kit. Dilution Buffer 1 is recommended for preparation of amniotic fluid samples. Dilution Buffer enables users to dilute EDTA plasma 8-fold and, thus, to further suppress potential matrix effects. Consequently, higher signal and better analytical characteristics are reached for EDTA plasma samples when using this new buffer.

1. INTENDED USE

The RD194070100R sHLA-G ELISA is a sandwich enzyme immunoassay for the quantitative measurement of soluble forms of human leukocyte antigen-G (sHLA-G).

Features

- **It is intended for research use only**
- The total assay time is about 20 hours
- The kit measures shedded HLA-G1 and HLA-G5 in EDTA plasma, amniotic fluid, or cell culture supernatant
- Calibrator is human native protein
- Assay format is 96 wells
- 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).
For stability of opened reagents see Chapter 9.

3. INTRODUCTION

Human leukocyte antigen-G (HLA-G) differs from the other MHC class I genes by its low polymorphism and alternative splicing that generates seven HLA-G proteins, whose tissue-distribution is restricted to normal fetal and adult tissues that display a tolerogeneic function toward both innate and acquired immune cells. Soluble HLA-G is an immunosuppressive molecule inducing apoptosis of activated CD8(+) T cells and down-modulating CD4(+) T cell proliferation.
Recently, using specific ELISA to analyse the presence of sHLA-G molecules in culture supernatants of early embryos obtained by in vitro fertilization (IVF) before transfer, several reports demonstrated that positive embryo implantations occurred with embryos secreting sHLA-G molecules. These breakthrough results indicate that sHLA-G ELISA can be a useful biochemical assay in addition to embryo morphology in embryo selection for transfer in IVF treatment if there are other embryos with the same morphology.
Furthermore, monitoring of sHLA-G in amniotic fluid and plasma of pregnant women may have an important prognostic value to recognize pathological situations.
Other interesting observations suggest that HLA-G molecules seem to be directly involved in transplant acceptation, and their analysis should be taken into consideration when monitoring transplant-patients status. In addition, soluble HLA-G plasma levels are increased in lymphoproliferative disorders or in patients suffering from malignant melanoma, glioma, breast and ovarian cancer.

Areas of investigation:
Immune Response, Infection and Inflammation
Reproduction
Transplantation

4. TEST PRINCIPLE

In the BioVendor sHLA-G ELISA, calibrators and samples are incubated in microplate wells pre-coated with monoclonal anti-sHLA-G antibody. After 16-20 hours incubation and washing, monoclonal anti-human β2-microglobulin antibody labelled with horseradish peroxidase (HRP) is added to the wells and incubated for 60 minutes with captured sHLA-G. 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. The absorbance is proportional to the concentration of sHLA-G. A calibration curve is constructed by plotting absorbance values against concentrations of calibrators, and concentrations of unknown samples are determined using this calibration curve.

5. PRECAUTIONS

- **For professional use only**
- Wear gloves and laboratory coats when handling immunodiagnostic materials
- Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled
- This kit contains components of animal origin. These materials should be handled as potentially infectious
- 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

6. 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

7. REAGENT SUPPLIED

Kit Components	State	Quantity
Antibody Coated Microtiter Strips	ready to use	96 wells
Conjugate Solution Conc. (100x)	concentrated	0.13 ml
Master Calibrator	lyophilized	2 vials
Conjugate Diluent	ready to use	13 ml
Dilution Buffer 1	ready to use	20 ml
Dilution Buffer 2	ready to use	13 ml
Wash Solution Conc. (10x)	concentrated	100 ml
Substrate Solution	ready to use	13 ml
Stop Solution	ready to use	13 ml

8. MATERIAL REQUIRED BUT NOT SUPPLIED

- Deionized (distilled) water
- Test tubes for diluting samples
- Glassware (graduated cylinder and bottle) for Wash Solution
- 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)

9. PREPARATION OF REAGENTS

All reagents need to be brought to room temperature prior to use.

Centrifuge liquid containing microtube vials before opening.

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 aluminium zip-sealed bag with desicant and seal carefully. Remaining Microtiter Strips are stable 3 months when stored at 2-8 °C and protected from the moisture.

Conjugate Diluent

Dilution Buffer 1

Dilution Buffer 2

Substrate Solution

Stop Solution

Stability and storage:
Opened reagents are stable 3 months when stored at 2-8 °C.

Assay reagents supplied lyophilized or concentrated:

sHLA-G Master Calibrator

Refer to the Certificate of Analysis for current volume of distilled water needed for reconstitution of calibrator!!!
Reconstitute the lyophilized Master Calibrator with distilled water just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam). The resulting concentration of the sHLA-G in the stock solution is **625 Units/ml**.

Prepare set of calibrators using the Dilution Buffer 1 (example for duplicates):

Volume of Calibrator	Dilution Buffer	Concentration
Stock	-	625.00 Units/ml
100 µl of stock	400 µl	125.00 Units/ml
250 µl of 125.00 Units/ml	250 µl	62.50 Units/ml
250 µl of 62.50 Units/ml	250 µl	31.25 Units/ml
250 µl of 31.25 Units/ml	250 µl	15.63 Units/ml
250 µl of 15.63 Units/ml	250 µl	7.81 Units/ml
250 µl of 7.81 Units/ml	250 µl	3.91 Units/ml

Prepared Calibrators are ready to use, do not dilute them.

Stability and storage:
Do not store the Calibrator stock solution and set of calibrators.

Conjugate Solution Conc. (100x)

Prepare the working Conjugate Solution by adding 1 part Conjugate Solution Concentrate (100x) with 99 parts Conjugate Diluent. Example: 10 µl of Conjugate Solution Concentrate (100x) + 990 µl of Conjugate Diluent for 1 strip (8 wells).

Stability and storage:
Opened Conjugate Solution Concentrate (100x) is stable 3 months when stored at 2-8 °C.
The working Conjugate Solution is stable 1 week when stored at 2-8 °C.

Wash Solution Conc. (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.

10. PREPARATION OF SAMPLES

The kit measures sHLA-G in EDTA plasma, amniotic fluid or cell culture supernatant. Samples should be assayed immediately after collection or should be stored at -20 °C (-70 °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.

Two diluents are available in the kit. Dilution Buffer 1 is recommended for preparation of amniotic fluid samples. Dilution Buffer enables users to dilute EDTA plasma 8-fold and, thus, to further suppress potential matrix effects. Consequently, higher signal and better analytical characteristics are reached for EDTA plasma samples when using this new buffer.

EDTA plasma samples:

Dilute samples 8x with Dilution Buffer 2 just prior to running the assay, e.g. 15 µl of sample + 105 µl of Dilution Buffer 2 for singlets, of preferably, 30 µl of sample + 210 µl of Dilution Buffer 2 for duplicates. **Mix well** (not to foam). Vortex is recommended.

Amniotic fluid samples:

Dilute samples 4x with Dilution Buffer 1 just prior to running the assay, e.g. 30 µl of sample + 90 µl of Dilution Buffer 1 for singlets, or preferably 60 µl of sample + 180 µl of Dilution Buffer 1 for duplicates. **Mix well** (not to foam). Vortex is recommended.

Stability and storage:
Samples should be stored at -20 °C, or preferably at -70 °C for long-term storage.
Do not store the diluted samples.

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 information at info@biovendor.com is assaying cell culture supernatants.

11. ASSAY PROCEDURE

1. Pipet **100 µl** of Calibrators, samples and Dilution Buffer (= blank), preferably in duplicates, into the appropriate wells. See *Figure 1* for example of work sheet.
2. **Incubate the plate at 2-8 °C for 16-20 hours, without shaking.**
3. Wash the wells 5-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add **100 µl** 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 5-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
7. Add **100 µl** 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 **25 minutes** at room temperature. The incubation time may be extended [up to 30 minutes] if the reaction temperature is below than 20 °C. No shaking!
9. Stop the colour development by adding **100 µl** 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 calibrator/s have absorbances above the upper limit of your microplate reader, perform a second reading at 405 nm. A new calibrator curve, constructed using the values measured at 405 nm, is used to determine sHLA-G concentration of off-scale calibrators 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 pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat four times. 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	**Calibrator 125.00**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
B	**Calibrator 62.50**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
C	**Calibrator 31.25**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
D	**Calibrator 15.63**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
E	**Calibrator 7.81**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
F	**Calibrator 3.91**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
G	**Blank**	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40
H	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41

Figure 1: Example of a work sheet.

12. CALCULATIONS

Most microplate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the mean absorbance (Y) of Calibrators against the known concentration (X) of Calibrators in logarithmic scale, using the four-parameter algorithm. Results are reported as concentration of sHLA-G (Units/ml) in samples.

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

The measured concentration of samples calculated from the calibration curve must be multiplied by their respective dilution factor, because samples have been diluted prior to the assay, e.g. 25 U/ml (from calibration curve) x 4 (dilution factor) = 100 U/ml.

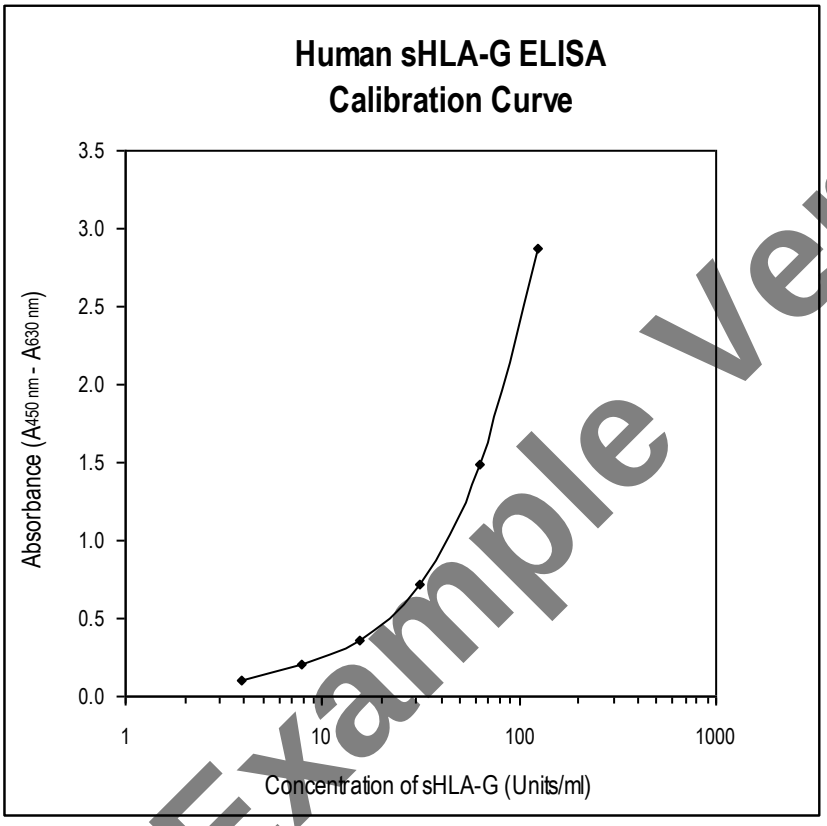

Figure 2: Typical Calibration Curve for sHLA-G ELISA.

13. PERFORMANCE CHARACTERISTICS

Typical analytical data of BioVendor sHLA-G 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: $A_{blank} + 3xSD_{blank}$, is calculated from the real sHLA-G values in wells and is 0.6 Units/ml.
*Dilution Buffer is pipetted into blank wells.

Limit of assay

Samples with absorbances exceeding the absorbance of the highest standard should be measured again with higher dilution. The final concentration of samples calculated from the standard curve must be multiplied by the respective dilution factor.

Presented results are multiplied by respective dilution factor.

Precision

Intra-assay (Within-Run) (n=8), for EDTA plasma samples diluted using the Dilution Buffer 2:

Sample	Mean (U/ml)	SD (U/ml)	CV (%)
1	327.75	16.01	4.9
2	658.77	23.02	3.5

Inter-assay (Run-to-Run) (n=5), for EDTA plasma samples diluted using the Dilution Buffer 2:

Sample	Mean (U/ml)	SD (U/ml)	CV (%)
1	342.31	22.32	6.5
2	909.63	67.39	7.4

Spiking Recovery

EDTA plasma samples were spiked with different amounts of sHLA-G and assayed (Dilution Buffer 2 was used as a diluent).

Sample	Observed (U/ml)	Expected (U/ml)	Recovery O/E (%)
1	99.22	-	-
	151.19	159.45	94.8
	191.28	216.77	88.2
	275.21	336.80	81.7
2	211.45	-	-
	316.31	329.00	96.1
	430.01	449.03	95.8
	620.35	686.54	90.4

Linearity

EDTA plasma samples were serially diluted with Dilution Buffer 2 and assayed.

Sample	Dilution	Observed (U/ml)	Expected (U/ml)	Recovery O/E (%)
1	-	175.59	-	-
	2x	93.30	87.80	106.3
	4x	44.65	43.90	101.7
2	-	271.78	-	-
	2x	136.16	135.89	100.2
	4x	60.74	67.95	89.4

Stability of samples stored at 2-8 °C

A significant decline in concentration of sHLA-G was observed in EDTA plasma after 7 days when stored at 2-8 °C. Therefore, we strongly recommend to store the samples at -20 °C, or preferably at -70 °C for long-term storage.

Effect of Freezing/Thawing

Significant changes in concentration of sHLA-G were observed in EDTA plasma samples after repeated freeze/thaw cycles. Therefore, it is strongly recommended to avoid unnecessary repeated freezing/thawing of the samples.

Sample	Number of f/t cycles	EDTA plasma (U/ml)
1	1x	134.64
	3x	233.28
	5x	206.72
2	1x	342.80
	3x	370.24
	5x	436.40
3	1x	625.60
	3x	860.80
	5x	890.80

Reference range

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

14. METHOD COMPARISON

The BioVendor sHLA-G ELISA has not been compared to any other commercial immunoassay.

15. 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 Calibrators, Quality Controls or samples

16. REFERENCES

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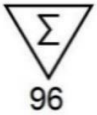
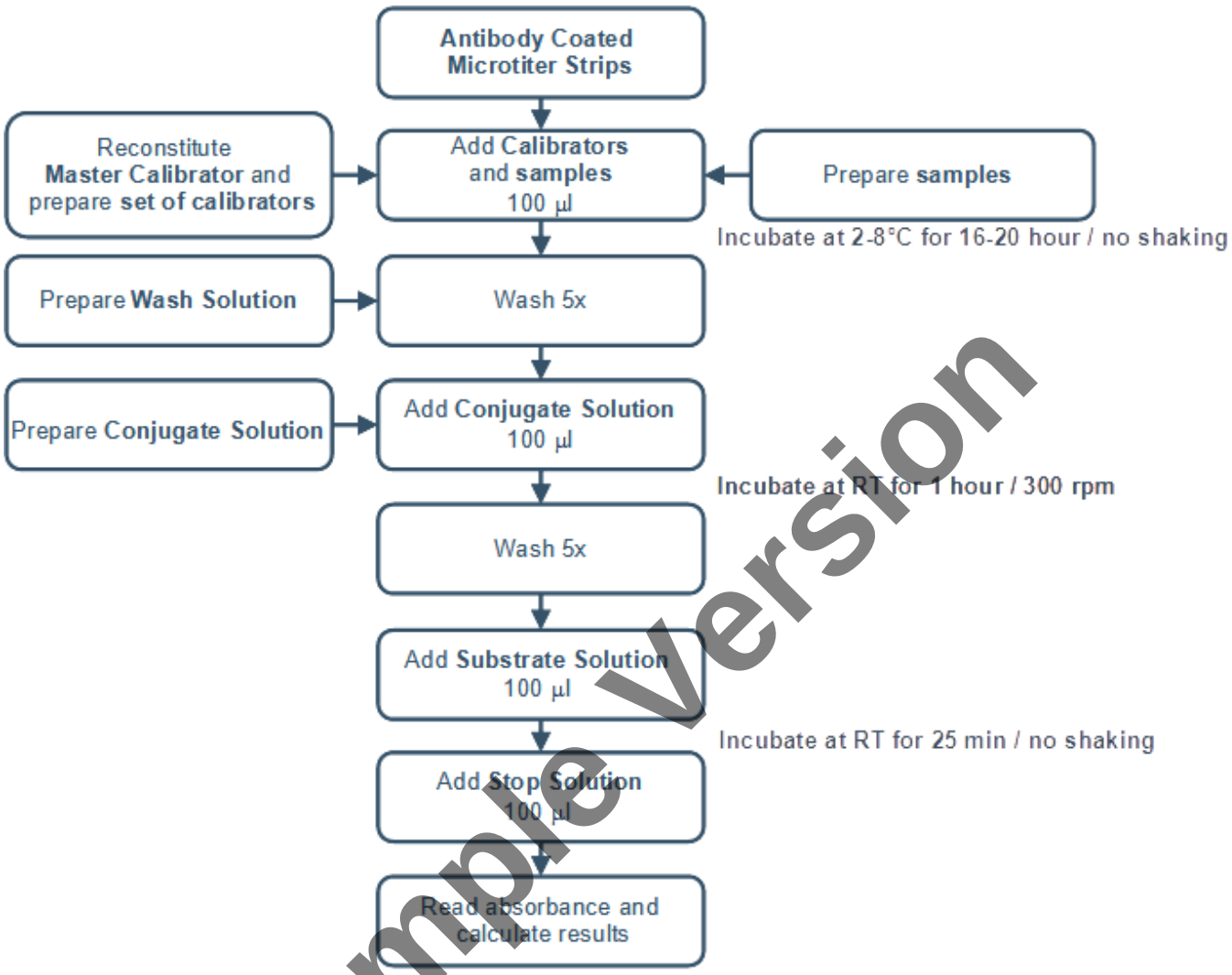
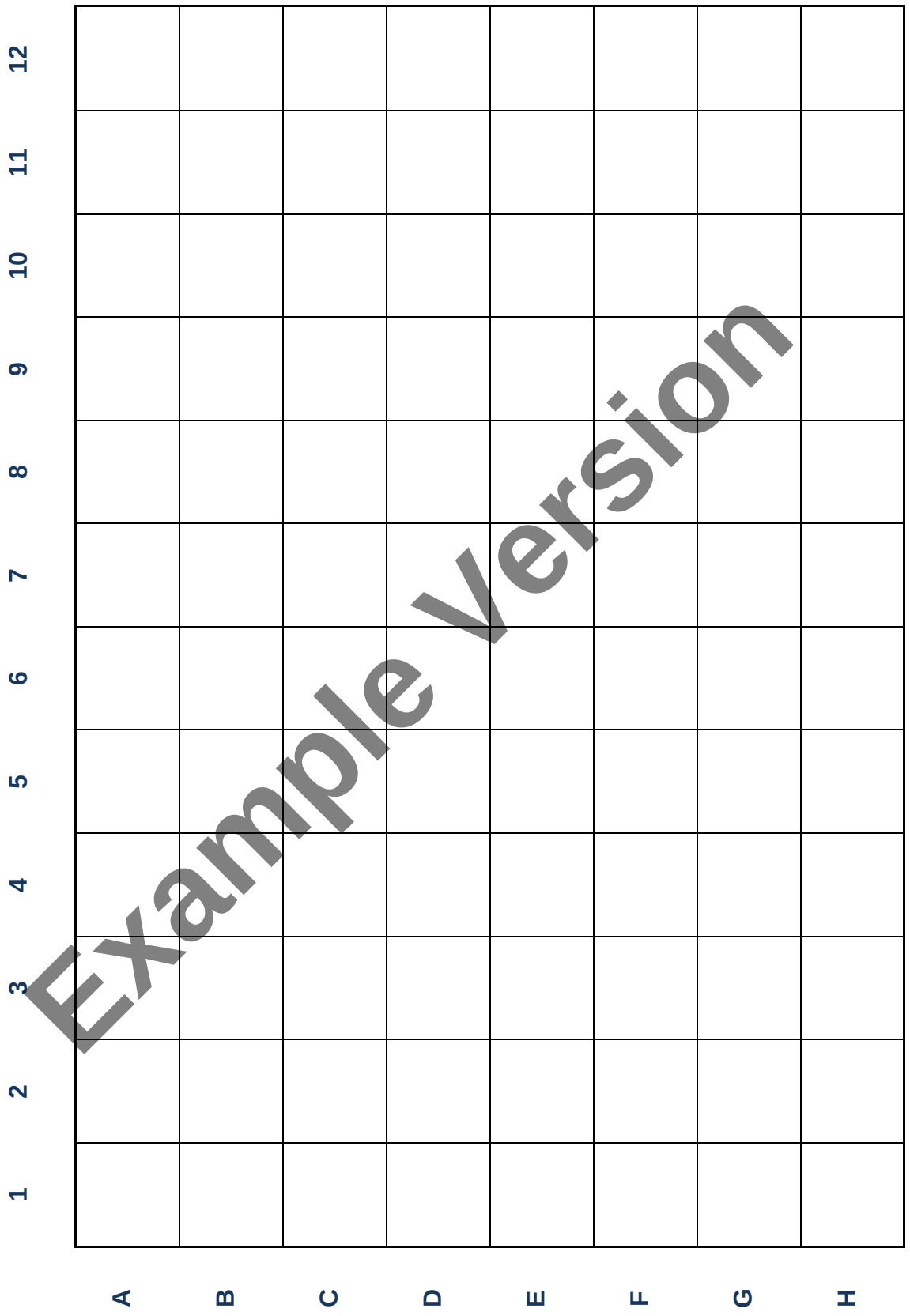
For more references on this product see our web pages at www.biovendor.com.

17. EXPLANATION OF SYMBOLS

REF	Catalogue number
LOT	Batch code
	Caution
	Use by date
	Temperature limit
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 96 tests
	Biological risks

18. ASSAY PROCEDURE - SUMMARY





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