BioVendor Group

ENG

Instructions for Use:

CANINE UROMODULIN ELISA

Catalogue number:

RBL023R

For research use only!

BioVendor R&D

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
	ENG.001.A
New edition	

1. INTENDED USE

The RBL023 Canine Uromodulin ELISA is a sandwich enzyme immunoassay for the quantitative measurement of canine uromodulin.

Features
- It is intended for research use only
- The total assay time is less than 3.5 hours
- The kit measures uromodulin in serum and urine
- Assay format is 96 wells
- Standard is canine native protein (from urine)
- 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

Uromodulin, also known as Tamm–Horsfall protein (THP) is the most abundant protein found in mammalian urine under physiological conditions selectively expressed by epithelial cells of the thick ascending limb of Henle's loop (TALH) [1, 2]. It is a glycosylphosphatidylinositol (GPI)-anchored apical membrane protein, released into the tubular lumen through proteolytic cleavage [3]. The normal urinary THP has a molecular weight of 80–90 kDa [1]. Approximately 30% of the molecular weight of THP is carbohydrates, mainly consisting of N-linked complex-type glycans with the most varied array of di-, tri- and tetra-antennary sugar structures [4]. Depending upon the species, urine uromodulin consists of around 640 amino acids.
It contains a very high number of cysteine residues that are completely engaged in disulfide bond formation, which is important for the conformation of the protein [1, 3, 5].
Canine uromodulin is most closely related to human and bovine THP with 80.7 and 79.9% identity to bovine and human THP, respectively [5]. In accordance to humans, the THP in the urine of dogs exists in a polymeric form with molecular weight above 5000 kDa which dissociates into monomeric molecules of around 100 kDa [6, 7].
It has been suggested that THP protein may be an important component of epithelia, which absorb sodium and chloride ions but are impermeable to water [6]. Uromodulin interacts with other molecules and cells including IL-1, IL-2, TNF, IgG, neutrophils, lymphocytes and monocytes [2, 8]. THP acts as a host defence factor against urinary tract infections, specifically targeting type 1 fimbriated Escherichia coli [9, 10]. Uromodulin also acts as an inhibitor of stone formation in healthy individuals by trapping crystals in the same manner. However, this function may be subverted under some circumstances and THP may facilitate crystal aggregation and then promote stone formation. It means that it has been proposed to play

a dual role as promoter or inhibitor of nephrolithiasis [11, 12].
A THP-like protein has been studied in dogs specifically because of the species' urinary excretion of vitamin A [10]. In contrast to humans, carnivores excrete vitamin A in urine as lipophilic retinol and retinyl esters. Therefore, THP is released in the urine of dogs to facilitate the excretion of retinol and retinyl esters [13, 14].
Previous reports have described urinary THP as biomarker for distal tubular dysfunction in dogs. Its potential use is illustrated by decreased urinary THP in dogs with chronic kidney diseases (CKD) and in dogs with urolithiasis [7, 11, 13, 15]. The high metabolic activity of the cells of the thick ascending limb of Henle's loop makes them particularly vulnerable to ischemic injury. Consequently, in acute kidney injury (AKI) caused by ischemic insults, evaluation
of THP as an early AKI biomarker is potentially valuable [13].
The study on the Bernese Mountain dogs concluded that THP together with the other identified urinary proteins may be valuable markers for the diagnosis of juvenile nephropathy [7].

Areas of investigation:
Renal diseases (canine)

4. TEST PRINCIPLE

In the BioVendor Canine Uromodulin ELISA, standards and samples are incubated in microtitration wells pre-coated with polyclonal anti-canine uromodulin antibody. After 60 minutes incubation followed by washing, biotin-labelled polyclonal anti-canine uromodulin antibody is added and incubated with the captured uromodulin for 60 minutes. After another washing, streptavidin-HRP conjugate is added. After 30 minutes incubation and the last washing step, the remaining 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 canine uromodulin. A standard curve is constructed by plotting absorbance values against uromodulin concentrations of standards and concentrations of unknown samples are determined using this standard 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 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

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
Biotin Labelled Antibody (100x)	concentrated	0.13 ml
Streptavidin-HRP Conjugate	ready to use	13 ml
Master Standard	lyophilized	2 vials
Dilution Buffer Conc. (10x)	concentrated	13 ml
Wash Solution Conc. (15x)	concentrated	50 ml
Substrate Solution	ready to use	13 ml
Stop Solution	ready to use	13 ml
Certificate of Analysis	–	1 pc

8. 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 5-1000 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.

Always prepare only the appropriate quantity of reagents for your test.

Do not use components after the expiration date marked on their label.

9.1 Assay reagents supplied ready to use:

9.1.1 Antibody Coated Microtiter Strips

Stability and storage:
Return the unused strips to the provided aluminum zip-sealed bag with desiccant and seal carefully. The remaining Microtiter Strips are stable for 3 months stored at 2-8 °C and protected from the moisture.

9.1.2 Streptavidin-HRP Conjugate

9.1.3 Substrate Solution

9.1.4 Stop Solution

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

9.2 Assay reagents supplied concentrated or lyophilized:

9.2.1 Canine Uromodulin Master Standard

Refer to the Certificate of Analysis for the current volume of Dilution Buffer needed for reconstitution of standard!!!
Reconstitute the lyophilized Master Standard 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 canine uromodulin in the stock solution is **10 ng/ml**.

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	Dilution Buffer	Concentration
Stock	-	10 ng/ml
300 µl of stock	300 µl	5 ng/ml
300 µl of 5 ng/ml	300 µl	2.5 ng/ml
300 µl of 2.5 ng/ml	300 µl	1.25 ng/ml
300 µl of 1.25 ng/ml	300 µl	0.63 ng/ml
300 µl of 0.63 ng/ml	300 µl	0.31 ng/ml
300 µl of 0.31 ng/ml	300 µl	0.16 ng/ml

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

Stability and storage:
Do not store the Standard stock solution and the set of standards.

9.2.2 Biotin Labelled Antibody (100x)

Dilute the Biotin Labelled Antibody concentrate hundred-fold with Dilution Buffer (e.g. 130 µl of Biotin Labelled Antibody concentrate + 12.87 ml of Dilution Buffer for 12 wells).

Stability and storage:
The reconstituted Biotin Labelled Antibody concentrate is stable for 1 week when stored at 2-8 °C. Do not store diluted Biotin Labelled Antibody solution.

9.2.3 Dilution Buffer Conc. (10x)

Dilute Dilution Buffer Concentrate (10x) ten-fold in distilled water to prepare a 1x working solution. Example: 13 ml of Dilution Buffer Concentrate (10x) + 117 ml of distilled water for use of all 96-wells.

Stability and storage:
The diluted Dilution Buffer is stable for 1 week when stored at 2-8 °C. Opened Dilution Buffer Concentrate (10x) is stable for 3 months when stored at 2-8 °C.

9.2.4 Wash Solution Conc. (15x)

Dilute Wash Solution Concentrate (15x) fifteen-fold in distilled water to prepare a 1x working solution. Example: 50 ml of Wash Solution Concentrate (15x) + 700 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 canine uromodulin in serum and urine.

Samples should be assayed immediately after collection or should be stored at -20 °C or -70 °C. Thoroughly mix thawed samples just prior to the assay and avoid repeated freeze-thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

An appropriate dilution should be assessed by the researcher in advance to batch measurement.

Recommended starting dilution for canine serum is 40x.
Dilute serum samples 40x with the Dilution Buffer just prior to the assay as follows:
Add 10 µl of sample into 390 µl of Dilution Buffer. Mix well (not to foam).
Vortex is recommended.

Recommended starting dilution for canine urine is 50 000x.
Dilute urine samples 50 000x with the Dilution Buffer just prior to the assay in **three** steps as follows:
Dilution A (20x):
Add 5 µl of sample into 95 µl of Dilution Buffer. Mix well (not to foam).
Vortex is recommended.
Dilution B (25x):
Add 5 µl of Dilution A into 120 µl of Dilution Buffer Mix well (not to foam).

Dilution C (100x):
Add 4 µl of Dilution A into 396 µl of Dilution Buffer Mix well (not to foam).

Vortex is recommended.

Stability and storage:
Serum samples should be stored at -20 °C, or preferably at -70 °C for long-term storage.
Do not store the diluted samples. Avoid repeated freeze/thaw cycles.

11. ASSAY PROCEDURE

1. Pipet **100 µl** of Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells. See Figure 1 for example of work sheet.
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 the final wash, invert and tap the plate strongly against paper towel.
4. Add **100 µl** of Biotin Labelled Antibody 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 the final wash, invert and tap the plate strongly against paper towel.
7. Add **100 µl** of Streptavidin-HRP Conjugate into each well.
8. Incubate the plate at room temperature (ca. 25 °C) for **30 min**, shaking at ca. 300 rpm on an orbital microplate shaker.
9. Wash the wells 3-times with Wash Solution (0.35 ml per well). After the final wash, invert and tap the plate strongly against paper towel.
10. 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.
11. Incubate the plate for **20 minutes** at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below 20 °C. Do not shake the plate during the incubation.
12. Stop the colour development by adding **100 µl** of Stop Solution.
13. Determine the absorbance of each well on 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 12**.

Note 1: 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 canine uromodulin 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 pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat twice. After the 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 10**	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	**Standard 5**	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	**Standard 2.5**	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	**Standard 1.25**	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	**Standard 0.63**	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	**Standard 0.31**	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	**Standard 0.16**	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	**Blank**	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

Figure 1: Example of a work sheet.

12. PERFORMANCE CHARACTERISTICS

Typical analytical data of BioVendor Canine Uromodulin ELISA are presented in this chapter.

12.1 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 canine uromodulin values in wells and is: 10 pg/ml.
* Dilution Buffer is pipetted into Blank wells.

12.2 Limit of assay

Results exceeding canine uromodulin level of 10 ng/ml should be repeated with more diluted samples. Dilution factor needs to be taken into consideration in calculating the canine uromodulin concentration.
Presented results are multiplied by respective dilution factor.

12.3 Precision

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

Sample	Mean (ng/ml)	SD (ng/ml)	CV (%)
Serum 1	140.65	6.34	4.5
Serum 2	65.94	2.16	3.3

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

Sample	Mean (ng/ml)	SD (ng/ml)	CV (%)
Serum 1	142.64	7.11	5.0
Serum 2	65.60	5.81	8.9

12.4 Spiking Recovery

Serum samples were spiked with different amounts of canine uromodulin and assayed.

Sample	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
Serum 1	37.77	-	-
	50.87	50.27	101.2
	63.08	62.77	100.5
	86.66	87.77	98.7
Serum 2	29.85	-	-
	39.87	42.35	94.1
	49.93	54.85	91.0
	73.47	79.85	92.0

Sample	Observed (µg/ml)	Expected (µg/ml)	Recovery O/E (%)
Urine 1	11.64	-	-
	22.13	21.02	105.3
	31.95	30.39	105.1
	50.59	49.14	103.0
Urine 2	45.76	-	-
	56.52	55.13	102.5
	69.48	64.51	107.7
	90.17	83.26	108.3

12.5 Linearity

Serum and urine samples were serially diluted with Dilution Buffer and assayed.

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
Serum 1	-	131.03	-	-
	2x	62.53	65.51	95.4
	4x	33.35	32.76	101.8
	8x	16.62	16.38	101.5
Serum 2	-	94.34	-	-
	2x	49.40	47.17	104.7
	4x	23.76	23.59	100.8
	8x	11.54	11.79	97.8

Sample	Dilution	Observed (µg/ml)	Expected (µg/ml)	Recovery O/E (%)
Urine 1	-	89.41	-	-
	2x	44.53	44.71	99.6
	4x	22.81	22.35	102.0
	8x	11.63	11.18	104.0
Urine 2	-	79.64	-	-
	2x	43.51	39.82	109.3

	4x	19.91	19.91	100.0
	8x	10.08	9.95	101.3

13. DEFINITION OF THE STANDARD

In this assay purified native protein from canine urine is used as the standard. It is a 64,48 kDa protein (calculated without glycosylation) consisting of 589 amino acid residues.

14. PRELIMINARY POPULATION AND CLINICAL DATA

Mean concentration of canine uromodulin in serum samples from 16 healthy donors (beagle dogs) was 75.3 ± 30.0 ng/ml (median 74.6 ng/ml). The data quoted in these instructions should be used for guidance only. It is recommended that each laboratory includes its own panel of control sample in the assay.

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 Standards, Quality Controls or samples

16. CALCULATION

Most microtiter plate readers perform automatic calculations of analyte concentration.
The Standards curve is constructed by plotting the 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 canine uromodulin (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).

The measured concentration of samples calculated from the standard curve must be multiplied by the respective dilution factor, because samples have been diluted prior to the assay, e.g. 0,63 ng/ml (from standard curve) x 40 (dilution factor) = 25,2 ng/ml.

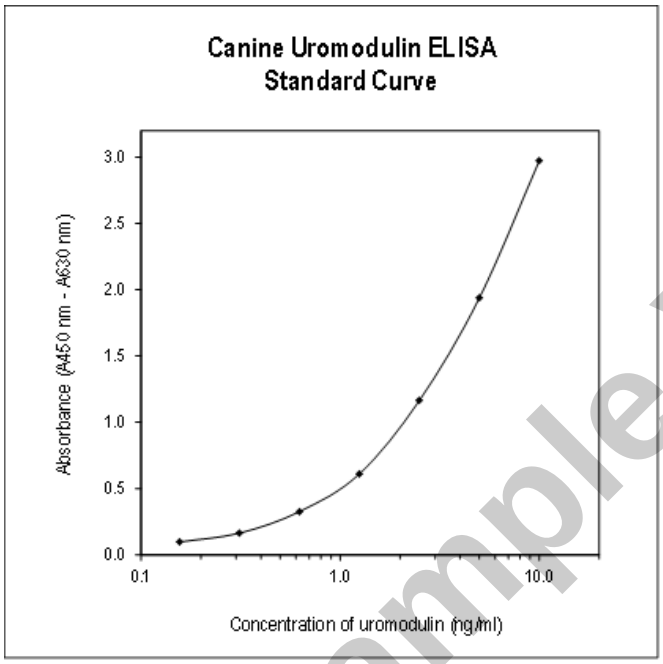

Figure 2: Typical Standard Curve for Canine Uromodulin ELISA.

17. REFERENCES

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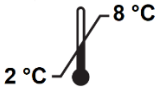
18. EXPLANATION OF SYMBOLS

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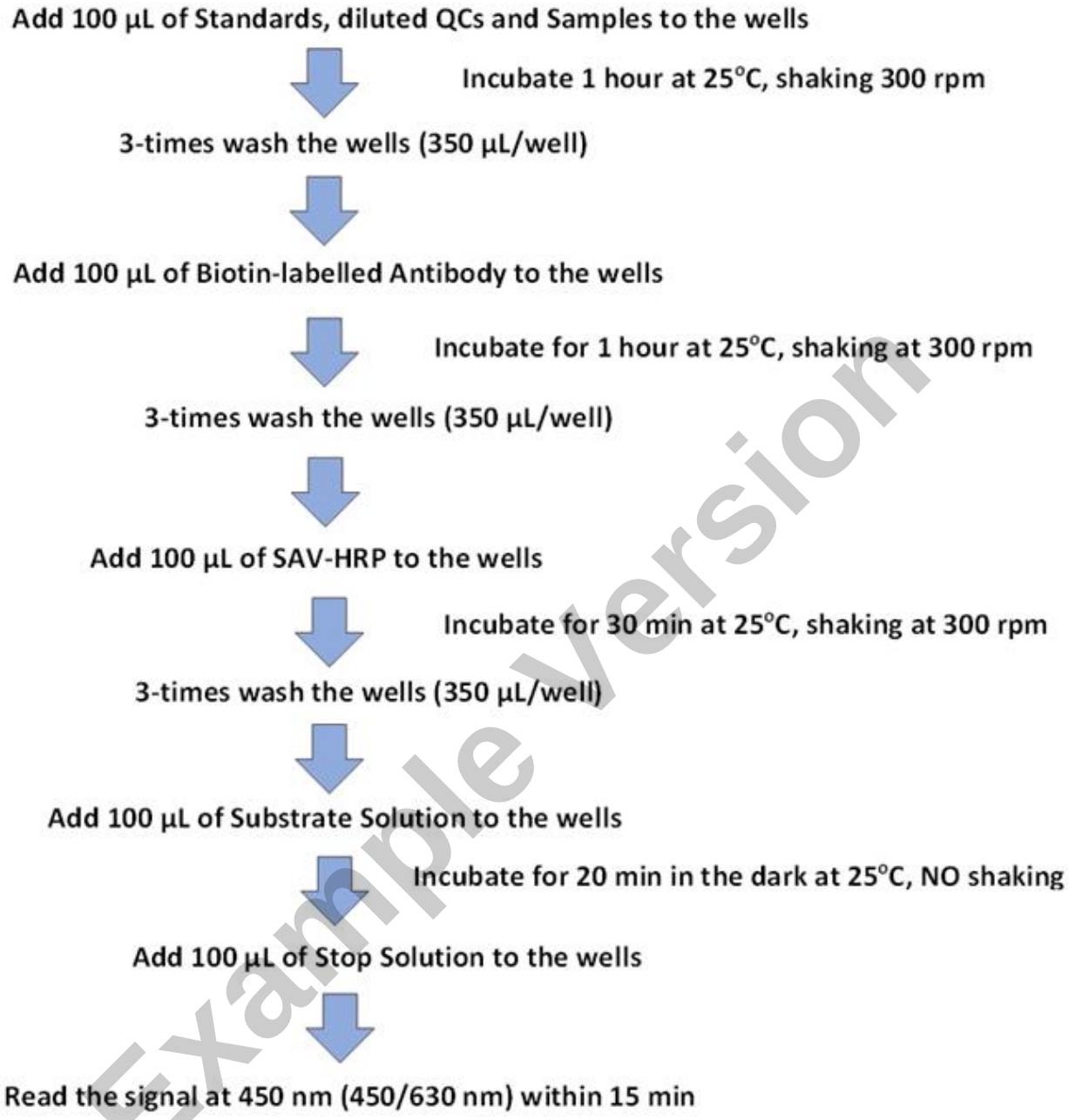
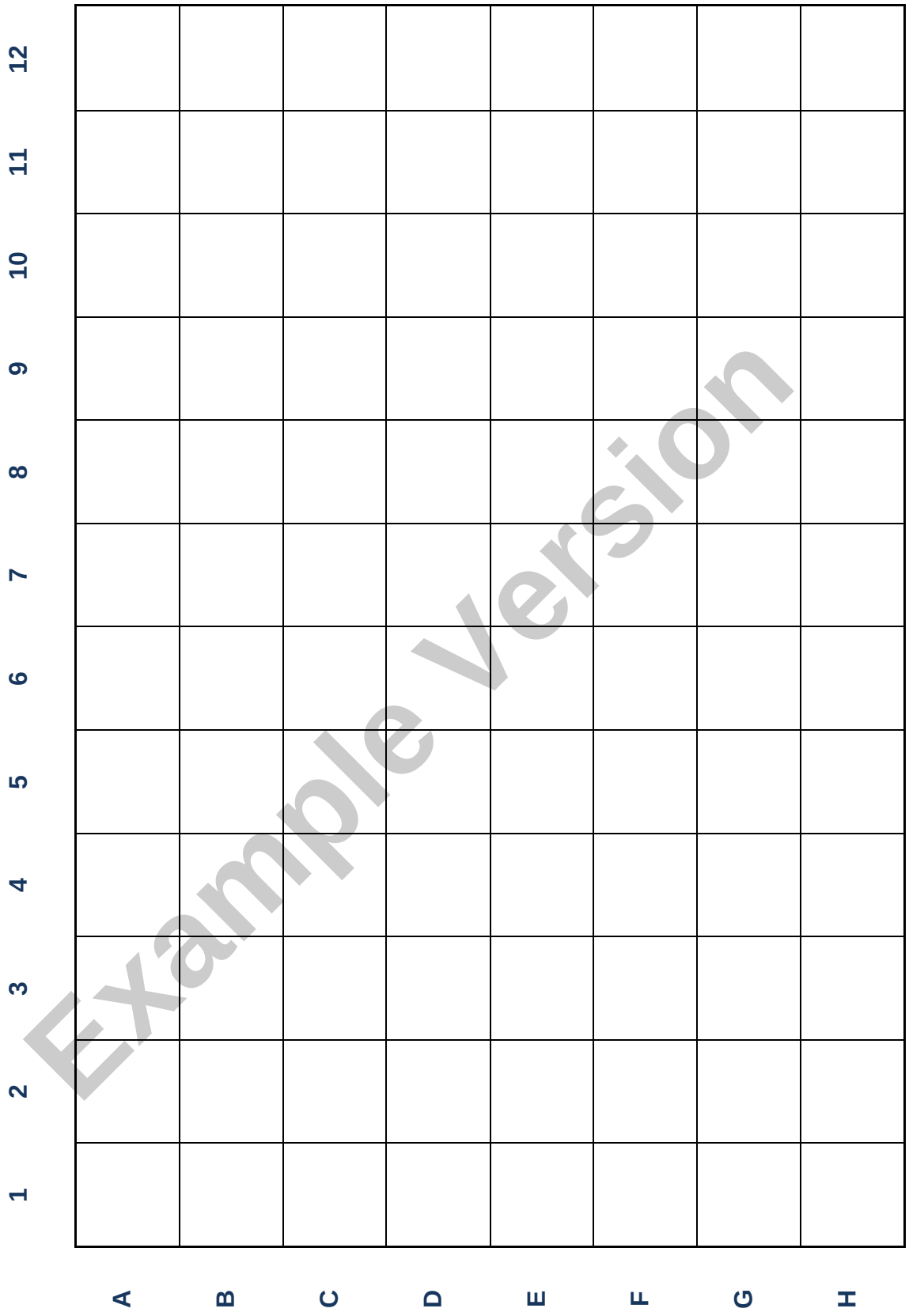
	Read electronic instructions for use - eIFU
	The content is sufficient for 96 tests
	Biological risks

19. ASSAY PROCEDURE - SUMMARY





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