Nu.Q® Discover H3.1 ELISA Assay - RUO

REF 1001-01-03

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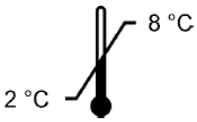

Store at 2-8°C

RUO For *Research Use Only – Not for Use in Diagnostic Procedures*

MANUFACTURER QUALITY CONTROL

All manufactured and commercialized reagents are under complete quality system starting from reception of raw material to the final commercialization of the product.
Each lot is submitted to a quality control and is only released on the market when conforming to the acceptance criteria.
The records relating to production and control of each single lot are kept within our company.

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1. INTRODUCTION

Nucleosomes are repeating subunits of DNA and histone proteins that constitute human chromatin. During cellular damage, such as apoptosis or necrosis, chromatin is fragmented into oligo- or mono- nucleosomes which can be released into the blood stream[1,2]. Histones and DNA are subject to a variety of epigenetic modifications.

2. PRINCIPLE OF THE TEST

The Nu.Q® Discover H3.1 ELISA Assay - RUO is a sandwich format assay. The sample is added with the assay buffer to the well coated with anti-H3.1 antibody. After a first incubation followed by a washing step, the detection antibody coupled to HRP is added. After a second incubation and washing step, the TMB substrate is added. The absorbance is read after addition of stop solution. The signal obtained is proportional to the concentration of H3.1 nucleosome present in the sample.

3. KIT COMPONENTS

3.1. Contents of the kit

All reagents are exclusively for *Research Use Only - Not for Use in Diagnostic Procedures*

Component	Symbol	Description	Volume
Antibody coated plate	CP1001	12 strips of 8 wells coated with monoclonal anti-H3.1 antibody against nucleosomes and provided on a carrier tray	1 plate
Wash buffer	WB1001	10X concentrated Wash buffer, containing preservative ProClin®300 and Tween 20 0.5%	2x 1 vial (30 mL)
Assay buffer	AS1001	Ready-to-use Assay buffer, containing CMIT/MIT preservative	1 vial (14 mL)
Standard curve Standard A→ F	SC2001-A SC2001-B SC2001-C SC2001-D SC2001-E SC2001-F	Lyophilised solutions containing H3.1 nucleosome to be used for the standard curve	6x 1 vial (1mL)
Kit controls 1 & 2 CONTROL +	KC1001-1 KC1001-2	Lyophilised solutions containing H3.1 nucleosome to be used as controls	2x 1 vial (1 mL)
HRP labelled detection antibody	DE1001	Ready-to-use solution of Anti-nucleosome antibody conjugated with horseradish peroxidase (HRP)	1 vial (14 mL)
Substrate solution	SU1001	Ready-to-use TMB (3,3',5,5'-Tetramethylbenzidine) substrate ⚠ **Note: the substrate solution is light sensitive**	1 vial (14 mL)
Stop solution	ST1001	Hydrochloric acid 1M ⚠ **Note: avoid any skin and eyes contact**	1 vial (14 mL)
Sealing film	N/A	Sealing films	3 Seals

3.2. Material required but not provided

- Deionized water.
- Adjustable or preset single-channel pipettes able to dispense 20 µL.
- Multi-channel pipettes able to dispense 50 µL to 200 µL.
- Disposable pipette tips.
- Disposable reagent reservoirs.
- Vortex mixer.
- Volumetric cylinders.
- Container for biohazardous waste.
- Microplate incubator with orbital shaking facility (shaker at 700 rpm).
- Microplate reader equipped with 450 nm filter
- Absorbent paper.
- Light-tight plate cover.

REMARK: For best assay efficiency, we recommend the use of a qualified calibrated pipettes and the use of corresponding tips according to supplier information, qualified calibrated shaker, and qualified calibrated reader.

4. STORAGE CONDITIONS & SHELF-LIFE

The kit must be stored at 2-8 °C.

At this temperature, the unopened and non-reconstituted reagents contained in the Nu.Q® Discover H3.1 ELISA Assay - RUO may be used up to the expiry date printed on the labels. Do not use reagents beyond this date.

5. PREPARATION OF THE REAGENTS

5.1. Standard curve and kit controls

The Standard curve and the Kit Controls 1 and 2 are supplied lyophilized. They must be reconstituted immediately before use.

Add 1 mL of deionized water to each bottle. Replace the stopper. Set a timer for 15 minutes to reconstitute. **Do not invert/vortex** the vials during the 15 minutes so that the cake has an opportunity to fully dissolve.Once the reconstitution of the standards and controls are complete, vortex the standards and kit controls for at least 5 seconds. Avoid the formation of foam while vortexing.

Once reconstituted, the standards and kit controls must be aliquoted by 100 µL (recommended volume) and stored at -80 °C (-65/85 °C) up to 4 weeks. Each aliquot once used, must be discarded directly. No freeze thaw cycles are permitted.

The concentration of the standard curve and the range of the kit controls is lot specific and is provided in the Certificate of Analysis.

5.2. Wash buffer

Dilute 10-fold. For example, 50 mL of 10X concentrated Wash buffer may be added to 450 mL deionized water to a final volume of 500 mL.

The diluted wash buffer may be stored at 2-8 °C for up to 14 days.

NB: It is possible to observe the presence of crystals in the concentrated wash solution. In this case, mix at room temperature until complete dissolution of crystals. For greater accuracy, dilute the whole bottle of concentrated wash solution, taking care to transfer completely the crystals, then mix until crystals are completely dissolved.

5.3. Coated microplate

Open the coated microplate only when it is at room temperature. Unused coated microwell strips should be sealed securely in the foil pouch containing desiccant and stored at 2-8 °C.

Once opened, the microplate is stable up to 14 days at 2-8 °C.

6. SPECIMEN

6.1. Specimen collection, preparation, and storage

The Nu.Q® Discover H3.1 ELISA assay - RUO has been validated for use on frozen Human K2 EDTA plasma samples. We advise to store these plasma samples at -80 °C (-65/-86 °C).

Avoid repetitive freezing and thawing of the plasma samples. A maximum of three freeze thaw cycles is permitted.

The following protocol is suggested to prepare the specimen:

- Draw 10 mL whole blood into K2 EDTA tube.
- Immediately invert each of vacutainer tubes 10 times gently.
- Blood sample must be processed **within 4 hours** of blood draw. Blood could be stored in the refrigerator (+2-8 °C) or kept at Room Temperature (15-25 °C) between blood draw and centrifugation.
- Centrifuge blood in vacutainer tube(s) for 10-15 minutes between 1500 and 3000 rcf with no braking at Room Temperature (15-25°C).
- Using a clean transfer pipette, carefully pipette off the plasma supernatant, taking care not to disturb the buffy coat layer.
- Leave at least a 1 cm gap between the pipette tip and the buffy coat (approximately 4-5 mL plasma can be obtained from 10 mL blood).
- Transfer into a labelled 2mL cryovial and store at -80°C (-65/-86 °C).

Before performing the measurement, the plasma samples must be thawed at room temperature (15-25 °C) without the use of extra heating and **must be vortexed and centrifuged 2 minutes at 14000 rcf. After centrifugation, transfer the supernatant to a clean tube without disturbing the pellet.**

Notes:
(1) Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay (see chapter 13.2 - Interferences).
(2) The supernatant transfer in a clean tube is mandatory as not transferring could affect the kit performances, even if the pellet is not visible.

6.2. Specimen Dilution

The reportable range of the Nu.Q® Discover H3.1 ELISA Assay - RUO is 22.7 ng/mL to 650 ng/mL.

The test must be performed on undiluted K2 EDTA plasma. Samples with concentrations above the reportable range cannot be quantified with accuracy. A concentration can be estimated by extrapolating values using a 4-parameter logistic function curve fitting.

7. PRECAUTIONS

The reliability of the results depends on correct implementation of good safety and hygiene principles including:

- Do not use reagents beyond the expiry date on the labels.
- Do not use damaged plate packaging.
- Please adhere strictly to the sequence of pipetting steps and reagent preparation steps provided in these instructions for use.
- Replace caps on reagents immediately after use. Do not switch caps.
- Do not allow the microplate to dry between the end of the washing operation and dispensing of the next reagent.
- Use a new pipette tip to dispense each patient specimen.
- Never use the same reservoir to distribute the HRP and substrate solution.
- Well washing is a critical step: adhere to the recommended number of washing cycles and make sure that all wells are filled and then completely emptied. Incorrect washing may lead to inaccurate results.
- Do not change the assay procedure. All indicated volumes must be performed according to the Instructions For Use.
- Plasma K2 EDTA samples should be left 30 minutes to warm to room temperature (15-25 °C) prior to assay without the use of extra heating.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond 15 minutes to avoid assay drift. If more than 15 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.

- Maximum precision is required for dispensation of the reagents.
- Plate readers measure vertically. Do not touch the bottom of the wells.
- Do not mix component from different kit lots.

8. HEALTH AND SAFETY INSTRUCTIONS

- All the reagents included in the kit are intended for "*Research Use Only*". Not for internal or external use in Humans or Animals or In Vitro Diagnostic use.
- This test kit should be handled only by qualified personnel trained in laboratory procedures and familiar with their potential hazards. Wear appropriate protective clothing, including lab coat, eye/face protection and disposable gloves and handle reagents and samples according to good safety and hygiene principles. Wash hands thoroughly after performing the test.
- Any equipment directly in contact with specimens and reagents as well as the washing solutions should be considered as contaminated products and treated as such.
- Some reagents contain preservative. Avoid release to the environment.
- Handling should be in accordance with the procedures defined by appropriate national biohazard safety guidelines or regulations.
- Do not pipette by mouth.
- The TMB substrate contains an irritant, which may be harmful if inhaled, ingested, or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted chlorohydric acid solution which is poisonous, corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.

9. TEST PROCEDURE

9.1. General remarks

- Follow the instructions carefully. Once started, all test steps should be completed without interruption.
- Use a new disposable plastic pipette tip for each control or sample to avoid cross contamination.
- Each reagent reservoir must be dedicated to a unique reagent. Never reuse or mix reservoirs that have contained different solutions.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred back into the original vials.
- Once used, store the reagents immediately at 2-8 °C. Avoid exposure to room temperature beyond the time required to perform the experiment.
- Avoid the exposure of reagent TMB Substrate to direct sunlight, metals or oxidants. Do not freeze the solution.

Always use all reagents from a single Nu.Q® Discover H3.1 ELISA Assay - RUO. The reliability of results may be affected by use of reagents from different test kits.

9.2. Assay procedure

○ Assay preparation

Each assay must include a complete standard curve (Standard A to F) and 2 kit controls allowing proper quantification of samples.

1. Carefully establish the plate design. All standard curve points, kit controls and samples should be run at least in duplicate.
2. Prepare the Standard curve, Kit controls and diluted Wash buffer (refer to section 5).
3. Allow the reagents to warm up for 30min to room temperature (15-25 °C) and homogenize each of them before use.
4. Vortex all plasma samples, standard curve points and kit controls to homogenize them.

Before performing the measurement, the plasma samples must be thawed at room temperature (15-25 °C) without the use of extra heating and must be **centrifuged 2 minutes at 14000 rcf. After centrifugation, transfer the supernatant to a clean tube without disturbing the pellet.**

○ Manual procedure

1. First allow all reagents to reach room temperature (15-25 °C) for at least 30 minutes before use.
2. **Before use, wash the wells 3 times with 200 µL of Wash Buffer.** Remove each time the excess solution by tapping the inverted plate on an absorbent paper towel (e.g. paper tissue or towel).
3. Dispense 80 µL of Assay Buffer into each well.
4. Dispense 20 µL of each sample, standard curve point or kit control into the appropriate wells according to your plate design. Use a new disposable pipette tip for each sample.
5. Cover the microplate with a sealing film. Press firmly to ensure a tight seal.
6. Incubate the microplate at 15 °C to 25 °C on a microplate incubator with orbital shaking at approximately 700 rpm during 150 minutes.
7. Remove the adhesive film. Empty the contents of all wells by decanting and tapping the microplate on an absorbent material (e.g. paper tissue or towel) and wash the wells 3 times with 200 µL diluted Wash Buffer. Following each washing step, remove the Wash buffer from the plate by decanting and tapping the plate on an absorbent material.
8. Add 100 µL of HRP labelled detection antibody into each well. Cover the plate with a sealing film and incubate at 15 °C to 25 °C for 90 minutes on a microplate incubator with orbital shaking at approximately 700 rpm.
9. Repeat the washing procedure described in step 7.
10. Add 100 µL of TMB substrate solution into each well. Allow the colour reaction to develop in the dark for 20 minutes at 15 °C to 25 °C with gentle shaking on a microplate incubator with orbital shaking at approximately 700 rpm in the dark (cover the plate with a light-tight cover, do not use adhesive film during this incubation)
11. After 20 min, stop the colorimetric reaction by adding 100 µL of Stop solution to each well. Use the same sequence and rate of dispensing as for the substrate solution. Mix by placing

the plate for 1 min on the microplate incubator with orbital shaking.

12. Within the next 5 minutes, determine the optical density (OD) of the wells at 450 nm using a microplate reader.

9.3. System adaptation

The volumes provided in the kit allow the final user to use an automated microplate washer. This adaptation step must be optimized and validated by the final user to ensure valid sample results.

10. QUALITY CONTROL

The mean value of the different controls must be within the specifications described in the table below:

Control	Acceptable range
Standard curve A	OD value < 0.20
Kit Control 1	See range in Certificate of Analysis
Kit Control 2	See range in Certificate of Analysis

Any value out-of-range for these controls invalidates the assay which must be performed again.

Each lab should assay controls at different levels of H3.1 nucleosome for monitoring assay performances. These controls should be treated as unknowns and values determined in every test procedure performed.

11. CALCULATION AND INTERPRETATION OF RESULTS

11.1. Calculation of the mean OD value and coefficient of variation for the samples

Before sample analysis, the mean absorbance and coefficient of variation of each sample must be calculated as follow:

$$\textbf{\textit{Mean OD}}\ (\overline{\textbf{\textit{OD}}}) = \frac{\textbf{\textit{OD duplicate 1}}(\textbf{\textit{OD 1}}) + \textbf{\textit{OD duplicate 2}}\ (\textbf{\textit{OD 2}})}{\mathbf{2}}$$

$$\textbf{\textit{Standard Deviation}}\ (\textbf{\textit{Sd}}) = \sqrt{\frac{(\textbf{\textit{OD 1}} - \overline{\textbf{\textit{OD}}})^2 + (\textbf{\textit{OD 2}} - \overline{\textbf{\textit{OD}}})^2}{\mathbf{2}}}$$

$$Coefficient\ of\ Variation\ (\%CV) = \frac{Sd}{OD} \times 100$$

Any sample result with a coefficient of variation above 20 % must not be considered as valid and must be re-assayed.

11.2. Interpretation of the results

Interpretation of the results must be performed by interpolating the mean OD value of each tested sample against the standard curve and expressing them in ng/mL.

To do so, the construction of the standard curve using of a 4-parameter logistic function curve fitting with a weighing factor $1/Y^2$ is required.

NB: Use of a computer assisted method (e.g. *Prism* (GraphPad)) is recommended.

11.3. Troubleshooting

If you have any questions or issues, please check our Frequently Asked Questions for processing the Nu.Q® ELISA Assays on https://eifu.volition.com/.

For more information about the correct handling of Nu.Q® ELISA products, please check video on:
https://volition.com/resources/press-room/video-gallery/how-to-use-the-nu-q-test.

12. ASSAY PERFORMANCE

12.1. Analytical specificity

The specificity of the test was assessed with the following analytes.

Analyte	Cross-Reactivity
H3.1 nucleosome	100 %
H3.1 depleted for the first 32 aminoacids mononucleosome	2,2 %
Histone H3.1	0 %
H3.3 mononucleosome	7,7 %
H3.3 depleted for the first 32 aminoacids mononucleosome	2.1 %

12.2. Recovery

Recovery of the test was assessed by addition of known concentrations of H3.1 nucleosome to low or medium level plasma samples. Each concentration of analyte added into each patient sample were assayed using three lots of manufactured kits in triplicate.

The mean recovery obtained for the three batches were respectively of 96%, 104% and 96%, with a mean recovery of 98.7%.

Sample concentration (ng/mL)	Added analyte (ng/mL)	Measured (ng/mL)	Recovery (%)
35,5	40	85,68	113
35,5	80	128,3	111
71,3	40.0	108.8	98
71,3	80.0	142.9	94
Mean			104

Recovery results obtained with manufactured batch n°2

12.3. Linearity

Linearity was evaluated based on CLSI guideline EP-6A, “Evaluation of the Linearity of Quantitative Measurement Procedures”. Samples were prepared by diluting a plasma sample with high level of H3.1 with a plasma sample with low level of H3.1 prior to assay. Each dilution was assayed in triplicates. The resulting mean concentrations were compared to predicted concentrations.

The mean linearity obtained for each of the three batches are summarized in the table below:

	Mean linearity on samples set 1	Mean linearity on samples set 2
Manufactured batch 1	89%	95%
Manufactured batch 2	78%	90%
Manufactured batch 3	86%	95%
Mean	84.3%	93.3%

-	Observed concentration (O) (ng/mL)	Expected concentration (E) (ng/mL)	O/E
100 % High	500,3	/	/
80 % High + 20 % Low	355,1	401,2	89 %
60 % High + 40 % Low	280,2	302,2	93 %
40 % High + 60 % Low	181,6	203,2	89 %
20 % High + 80 % Low	87,8	104,2	84 %
100% Low	5,1	/	/
		Overall mean	89 %

Linearity results obtained with manufactured batch n°1 – sample set 1

12.4. Analytical sensitivity

The different analytical sensitivity parameters were evaluated following the CLSI guideline EP17A-2, “Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures”.

- **Limit of Blank (LoB)**

The Limit of Blank of the assay corresponds to the concentration below which analyte-free plasma samples are found with a probability of 95 %.

It was evaluated on three manufactured lots and defined at a concentration of 3.4 ng/mL.

- **Limit of Detection (LoD)**

The Limit of Detection of the assay is the lowest analyte concentration which can be detected (above the LoB with a probability of 95 %).

It was evaluated on three manufactured lots and defined at a concentration of 9.1 ng/mL.

- **Limit of Quantification (LoQ)**

The Limit of Quantification of the assay is the lowest analyte concentration that can be reproducibly measured with a CV at 20 %.

It was evaluated on three manufactured lots and defined at a concentration of 22.7 ng/mL.

12.5. Precision

Precision was determined using a modified protocol of the CLSI guideline EP5-A2 “Evaluation of Precision Performance of Quantitative Measurement Methods”. Experiment was performed on three manufactured batches. All samples were run in duplicate twice a day for minimum of 10 days per 2 operators.

The precision data are summarized in table below:

	N	Mean (ng/mL)	Within run precision		Total precision	
			SD	CV %	SD	CV %
Kit Control 1	80	33,6	2,3	6,8 %	4,1	12,1 %
Kit Control 2	80	298,8	12,7	4,2 %	25,5	8,5 %
IQC 1	80	15,1	1,8	11,8 %	2,9	19,3 %
IQC 2	80	106,5	7,5	7,1 %	9,8	9,2 %
IQC 3	80	236,4	17,3	7,3 %	23,8	10,1 %
IQC 4	80	487,3	40,2	8,3 %	55,7	11,4 %

Precision results obtained with manufactured batch n°1

13. LIMITATIONS OF USE

13.1. Limitation of the procedure

Reliable and reproducible test results can only be ensured when the test is performed in complete compliance with the present instructions and according to the applicable good safety and hygiene principles.

The wash procedure is critical. Insufficient washing will result in poor precision and false negative results.

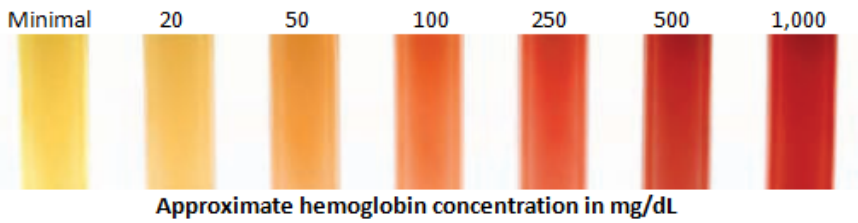
The assay must be performed in a laboratory with a temperature between 15-25 °C.

13.2. Interferences

The following potential interfering substances were tested and have shown not to interfere on the assay when present below the concentrations defined in the following table:

Interfering substance	Threshold concentration
Triglycerides	3000 mg/dL
Haemoglobin	500 mg/dL
Bilirubin, unconjugated	20 mg/dL
Bilirubin, conjugated	20 mg/dL
Protein (total)	8 g/dL
Cholesterol	300 mg/dL

Haemoglobin level can in a first place been estimated visually using the color scale below. For more precision, this visual check can be completed by an actual measurement of the hemoglobin level.



14. REFERENCES

1. Holdenrieder S. *et al.* (2001); Clin Chem Lab Med. “Nucleosomes in serum as a marker for cell death”, 39(7):596-605.
2. Silva MT. (2010); FEBS Lett. “Secondary necrosis: The natural outcome of the complete apoptotic program”, 584 (22):4491-9.
3. Fullgrabe J. *et al.* (2011); Oncogene “Histone onco-modifications”, 30(31):3391-403.

15. TECHNICAL SUPPORT

Any technical question or complaint related to this product can be submitted by email to EMEAcustomerservices@volition.com or sent directly using the form available on https://volitionrx.com/contact

Whenever applicable, please provide the reference of the product, lot number, description of the problem and test results.

16. SYMBOLS

RUO	Research Use Only device
REF	Catalogue number
LOT	Lot number
	Use by date
	Date of manufacturing
	Manufacturer
	Read instructions before use
	Store between 2 and 8 °C
	Number of tests
	Do not reuse
	Corrosive
	Keep away from sunlight
Symbols of the kit components supplied	