HCV Quantification Kit

USER MANUAL

For *in vitro* Diagnostic Use

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Contents

1. PRODUCT DESCRIPTION

Bosphore® HCV Quantification Kit detects and quantitates Hepatitis C Virus RNA in human serum and plasma, encompassing all the HCV genotypes. A region within the 5′UTR is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition and extraction. The amplification data of the internal control is detected with the HEX filter. The internal control can be added either during RNA extraction or PCR step.

2. CONTENT

Bosphore® HCV RNA Quantification Kit is composed of Real-Time RT PCR reagents and quantitation serum standards which have been calibrated against WHO International Standard (NIBSC Code 06/102):

Component	REAGENT	100 Reactions	50 Reactions	25 Reactions
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Master Mix	(2640 µl)	(1320 µl)	(660µl)
3	Internal Control	(560 µl)	(280 µl)	(140 µl)
4	Positive Control	(70 µl)	(35 µl)	(18 µl)
5	Standard 1 (1 x 10^6) IU/ml	(880 µl)	(880 µl)	(440 µl)
6	Standard 2 (1 x 10^5) IU/ml	(880 µl)	(880 µl)	(440 µl)
7	Standard 3 (1 x 10^4) IU/ml	(880 µl)	(880 µl)	(440 µl)
8	Standard 4 (2 x 10^3) IU/ml	(880 µl)	(880 µl)	(440 µl)

3. STORAGE

Bosphore® HCV Quantification Kit PCR reagents should be stored at -20°C. Repeated thawing and freezing (more than 3 times) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min. and the detection mix components should not be exposed to light or air more than necessary, vials must be kept closed except during pipetting. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483, Montania® 484 or Montania® 4896 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and HEX filters (such as iCycler, iQ5, CFX96–BioRad, LightCycler 480-Roche, 7500 Real-Time PCR System-ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGene 9600-Bioer, Rotorgene 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes, PCR plates or strips
- Magnesia® 16 Nucleic Acid Extraction System and Magnesia® Viral Nucleic Acid Extraction Kit, or Magrev®24 stand and Magrev® Viral DNA/RNA Extraction Kit, or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot and Magnesia® 2448 Viral DNA/RNA Extraction Kit, or Bosphore® Viral DNA Extraction Spin Kit (Anatolia Geneworks), or other high quality viral RNA extraction kits and systems
- Deep freezer (-20°C)
- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes

- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20ºC.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health related risks.
- Serum/plasma samples (including the standards) should be handled with extreme caution, suitable class microbiological safety cabinet should be used: Physical contact with pathogens should be avoided by; wearing lab coats and gloves, no allowance for eating or drinking within the workspace, prevention of unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid isolation step; including the serum samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual, by personnel specially trained to perform in vitro diagnostic procedures.

7. PATHOGEN

Causative Agents

Hepatitis C virus is a hepacivirus of the Flaviviridae family of viruses that causes Hepatitis C in humans. It is a small, enveloped, 9.6kb single-stranded RNA virus that is classified into six main genotypes (1-6) with more than one hundred different subtypes. [1]

Epidemiology

It is estimated that HCV has a worldwide prevalence of 3% affecting around 180 million people with between 3 to 4 million new infections each year. The vast majority of infected people (70-90%) develop chronic infection. Though chronic infection may be asymptomatic, it is a leading cause of chronic liver diseases, including cirrhosis in between 20 to 50% of patients. Treatment may be effective in 10-50% of patients depending on the applied therapy. [2]

Modes of Transmission:

Hepatitis C is believed to be spread through contact with infected blood. However, unlike many other blood borne viruses, HCV may be transmitted even through indirect sources like a used razor, making HCV more transmissible than other blood borne viruses –including HIV. Common routes of transmission include transfusion of blood products, intravenous and percutaneous drug and needle use, work accidents among healthcare workers and any other blood to blood contacts, such as sexual practices and from mother to newborn (maternal-infant transmission). Statistical studies have revealed no risk factors for HCV transmission in the activities of daily living (sneezing, coughing, hugging, etc.). [2], [3]

8. METHOD

Bosphore® HCV Quantification Kit is based on the Real Time RT PCR method. HCV genetic material is amplified by reverse transcription technique since it is composed of RNA. RT-PCR, which is also referred as RNA PCR, is a two-step reaction. First, complementary DNA is synthesized from RNA by reverse transcription and then complementary DNA is amplified by standard PCR. The primer binds to the target RNA region in RT-PCR and RNA-DNA double strand is synthesized by reverse transcriptase enzyme using the RNA template for complementary DNA. Afterwards, standard PCR continues.

Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis. In addition to these components, in RT PCR reverse transcriptase is added to the reaction and cDNA synthesis from the RNA template is acquired.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The assay utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hybridization probe during the extension phase of PCR.

The probe is labeled at the 5′ end with a fluorescent 'reporter' molecule, and at the 3′end with another fluorescent molecule that acts as a 'quencher' for the 'reporter'. When the two fluorophores are in close proximity, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, fluorescence signal can be detected.

The fluorescence generated by the reporter increases as the PCR product is accumulated; the point at which the signal rises above background level and becomes distinguishable, is called the threshold cycle (C_T). There is a linear relationship between the log of the starting amount of a template and its threshold cycle, thus starting amount of unknown templates can be determined using standard curves constructed using C_T values of the known starting amounts of target templates.

Bosphore HCV Quantification Kit employs multiplex PCR, and an internal control is incorporated into the system in order to control the isolation procedure and to check for possible PCR inhibition. HCV RNA (cDNA) and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the HCV amplification is detected by a probe labeled at the 3′ end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5′ end with a different reporter molecule, HEX) through the HEX channel.

9. PROCEDURE

While being used in combination with fully automated Magnesia 2448 Nucleic Acid Extraction & PCR Setup Robot all the user has to do is to place the kit within the worktable and start the HCV protocol. No manual processing is neccessary in case of automated use.

9.1. RNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System and Magnesia® Viral Nucleic Acid Extraction Kit, or Magrev®24 stand and Magrev® Viral DNA/RNA Extraction Kit, or Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot and Magnesia® 2448 Viral DNA/RNA Extraction Kit, or Bosphore® Viral DNA Extraction Spin Kit (Anatolia Geneworks), or other high quality viral RNA extraction kits and systems are used with Bosphore® HCV Quantification Kit. The RNA isolation should be performed according to the manufacturers' instructions. The amount of internal control that should be used during isolation for each system is 5 µl. The external quantitation standards are provided as serum, so that they undergo the same steps as the patient samples, starting from RNA isolation.

9.2. Kit Components

9.2.1. PCR Master Mix

PCR Master Mix is a high performance master mix that contains all the neccessary reagents for RT-qPCR of RNA templates; HotStarTaq DNA Polymerase, Reverse Transcriptase Mix, RT-PCR Buffer, dNTPs, the HCV-specific forward and reverse primers and a dual-labeled probe, and the internal control-specific forward and reverse primers and a dual-labeled probe. It is highly resistant to PCR inhibitors, and the cDNA synthesis and PCR amplification occur within the same tube sequentially without opening the tubes between procedures.

9.2.2. Internal Control

An internal control is included in the kit to control RNA isolation and PCR inhibition. The internal control is a synthetic DNA molecule. It is added into the serum/plasma, proteinase K and carrier RNA mixture during DNA isolation, to control the isolation efficiency and PCR inhibition. The amount of IC that should be added during isolation is 5 µl per serum/plasma sample. Alternatively, the internal control can be added directly into the PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.2 µl of internal control should be added for each reaction into the master mix. Lack of internal control amplification in the FAM negative samples, may indicate a problem in isolation or PCR inhibition. In this case, isolation and PCR should be repeated. In samples that contain a high viral load,

internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

HCV(FAM)	Internal Control (HEX)	Interpretation
+	+	Sample positive
-	+	Sample negative
+	-	Sample positive
-	-	Repeat the test!

9.2.3. Positive Control

The positive control contains HCV RNA. It can be included in the PCR to test the efficiency of the PCR exclusively. The threshold cycle for the positive control is given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

9.2.4. Quantitation Standards

The quantitation serum standards are calibrated by WHO International Standard (NIBSC Code 06/100).

9.3. Preparing the RT-PCR

All four external quantitation standards should be added into the PCR reaction together with the samples and the negative control (PCR-grade water). Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

PCR Master Mix	24 µl
Sample RNA (Standard, Negative/Positive Control)	16 µl
Total Volume	40 µl

Pipette 24 µl of the master mix into the PCR tubes or strips, and add 16 µl of RNA (sample/ standard/positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

9.4. Programming the Real-Time PCR Instrument

The thermal protocol for Bosphore® HCV Quantification Kit is composed of two-steps; an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a final hold. The real-time data is collected at the second step of the amplification cycle.

Reverse Transcription	50°C	30:00 min.	
Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	50 cycles
Annealing (Data Collection)	55°C	01:20 min.	
Synthesis	72°C	0:15 min.	
Hold	22°C	05:00	

To start a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and HEX),
- Identify unknown samples, standards, positive and negative controls, assign quantitative values to the standards,
- Select the correct thermal protocol.
- Start the protocol

10. ANALYSIS

By the end of the thermal protocol, the Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold. Example of an amplification curve is given in Fig. 1.

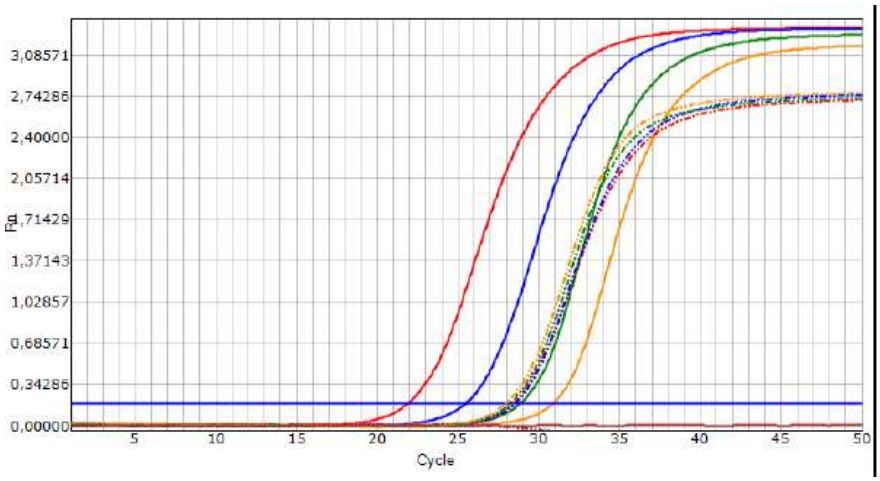

Fig. 1: Amplification Curve of a Bosphore® HCV test

The standard curve is plotted using the data obtained from the defined standards, with the axes Ct-Threshold Cycle and Log Starting Quantity. Example of a standard curve is given in Fig. 2.

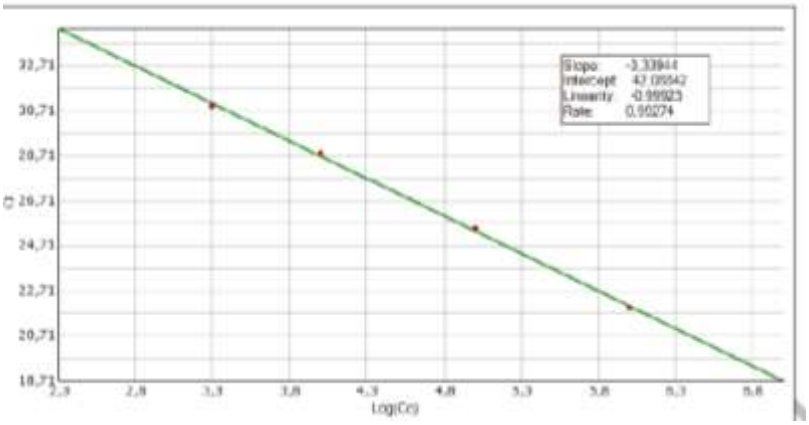

Fig.2: Standard Curve of a Bosphore® HCV test

Analysis of the results should be performed by trained personnel who have received the required training for analyzing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, and if the system's software allows pulling down the threshold as much as possible in order to detect low positive samples, attention should be paid to keep the threshold line above the background and to keep the correlation coefficient at the maximum possible value (and within its acceptance criteria. The table below displays the acceptance criteria for Bosphore® HCV Quantification Kit.

Component/Parameter	Cycle Threshold (C_T)
Standard 1	22±2
Standard 2	25±2
Standard 3	28.5±2
Standard 4	31±2
Positive Control	30±4
Correlation Coefficient	>0.970
PCR Efficiency*	>%80

* PCR efficiency is calculated by the following formula: $10^{(-1/\text{slope})}$-1 x100

Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

The quantitative results of the test contain the calculated starting quantities of the unknown samples in each tube. The samples that cross the threshold in FAM Channel are displayed with a calculated starting quantity, samples that do not cut the threshold are displayed as "No Ct". These samples are regarded as negative or having a viral load below the detection limit of the assay. For these undetectable samples, the HEX data of the internal control should also be checked to avoid false negative results

The following table shows the possible results and their interpretation:

Signal detected in FAM filter pair	The sample contains HCV RNA, the result is positive	No need to check the internal control since the sample is positive (high positive samples may suppress the signal from the internal control)
No signal in FAM, signal in HEX	The HCV RNA in the sample is not detectable	Signal from HEX filter pair rules out the possibility of PCR inhibition
No signal in FAM and HEX	The diagnosis is inconclusive	No signal in HEX points out to PCR inhibition or to a problem in RNA isolation

In rare cases of PCR inhibition due to medication or other PCR inhibitors in the sample, we recommend to repeat the test of inhibited samples, by freezing and thawing the RNA samples and using them in the PCR after diluting them 1:2 or 1:4 with dH_2O. (Caution: The dilution factor must be taken into account while reporting the Real-Time PCR quantitative results.)

11. SPECIFICATIONS

11.1. Sensitivity

Analytical sensitivity may be expressed as the limit of detection: i.e. the smallest amount of the target marker that can be precisely detected. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value. The analytical sensitivity or detection limit for NAT assays is expressed by the 95% positive cut-off value.

The analytical detection limit for Bosphore® HCV Quantification Kit was found to be $1.2x10^1$ IU/ml. The sensitivity was determined using Magrev® Viral DNA/RNA Extraction Kit with Magrev® 24 Nucleic Acid Extraction Stand (Anatolia Geneworks®), testing serial dilutions of serum samples calibrated with the WHO International Standard Hepatitis C Virus for Nucleic Acid Amplification Techniques (4th WHO International Standard) (NIBSC Code 06/102). The dilutions were subjected to viral RNA extraction and Real-Time PCR in different runs and in replicates. The results were analyzed by probit method.

The detection limit in consideration with the isolation for Bosphore HCV Quantification Kit was found to be $1.95x10^1$ IU/ml IU/ml using the Magnesia 2448® Viral DNA/RNA Extraction Kit and Magnesia® 2448 Nucleic Acid Extraction & PCR Setup Robot (Anatolia Geneworks®).

The detection limit in consideration with the isolation for Bosphore HCV Quantification Kit was found to be $2.5x10^1$ IU/ml IU/ml using the Magnesia® 16 Nucleic Acid Extraction System and Magnesia® Viral Nucleic Acid Extraction Kit (Anatolia Geneworks®).

11.2. Genotype Detection

Efficiency of detecting and quantitating different genotypes were ensured both by sequence comparison analysis and Real-Time PCR assays using QCMD Hepatitis C virus RNA EQA Programme 2011, 2012, 2013, 2014 and 2015 panel members and natural clinical samples (containing samples with HCV genotypes 1a, 1b and 3a) and the Worldwide HCV Performance Panel WWHV302(M) (Seracare). The following genotypes of the genotype panel were tested and found positive:

WWHV302(M) Panel Member	Genotype	HCV(FAM)
1	1b	+
2	1a	+
3	1b	+
4	2a/2c	+
6	3b	+
8	3a	+
10	4	+
11	4	+
12	5a	+
14	6a	+

11.3. Linear Range

The linear range of Bosphore® HCV Quantification Kit was determined to be from $1x10^1$ IU/ml to at least $1x10^9$IU/ml. In order to assess the linear range, a serum dilution series which has been calibrated against the WHO International Standard for HCV RNA NAT assays, (NIBSC Code 06/100) was analyzed by viral RNA extraction and Real-Time PCR. The standard curve correlation coefficient was found to be 0.995.

11.4. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of HIV, HDV, HBV, West Nile Virus, Dengue Virus with known high positivity were tested, and found negative.

11.5. Reproducibility and Precision

Reproducibility data was obtained by the analysis of one of the quantitation standards of the Bosphore® HCV Quantification Kit. Test was performed in at least 4 replicates by 3 different operators, on multiple days, using 3 different lots. The resulting data is given in Table 1 and Table2:

Table 1: Reproducibility Data.

HCV (10^4 IU/ml)	Standard deviation	Variance	Coefficient of variation [%]
Intra-assay Variability N=4	0.03	0.001	0.11
Inter-lot Variability N=3	0.29	0.08	0.92
Inter-operator Variability N=3	0.26	0.08	0.92
Total Inter-assay Variability N=5	0.25	0.06	0.81

Table 2: Precision Data.

HCV (10^4 IU/ml)	Measured Quantity (MQ) IU / ml	Standard Deviation (MQ)	Coefficient of variation [%] (MQ)	Threshold Cycle (Ct)	Standard Deviation (Ct)
Intra-assay Variability N=4	10852,5	267,87	2,46	31,24	0,03
Inter-lot Variability N=3	11072,17	1074,97	9,70	31,41	0,29
Inter-operator Variability N=3	9290,58	1354,50	14,57	31,53	0,26
Total Inter-assay Variability N=5	10047,15	1596,95	15,89	31,52	0,25

11.6. Diagnostic Evaluation

The diagnostic evaluation was initially performed by testing a total of 213 HCV negative and 71 HCV positive serum and plasma samples which have been previously analyzed using Roche Diagnostics Elecsys 2010, Roche COBAS Amplicor HCV RNA Monitor v2.0, Bayer Versant HCV RNA v3.0, Abbott HCV RNA m2000, Roche HCV RNA Taqman and Artus HCV RG RT-PCR Kit. All of the negative samples were found negative, and all of the positive samples were found positive with Bosphore® HCV Quantification Kit.

11.7. Calibration Against WHO Standard

Quantitation Standards were calibrated against the WHO HCV RNA International Standard (NIBSC Code 06/100 and 06/102). 1 IU was found to be equal to 3± 0,2 copies/ml .

12. REFERENCES

1. By K. E. Nelson, C. Williams, and N. Graham., Infectious Disease Epidemiology: Theory and Practice, July 15, 2000 , p :923-926
2. Theodore Sy and M. Mazen Jamal, Epidemiology of Hepatitis C Virus (HCV) Infection, Int J Med Sci. 2006; 3(2), p:41–46.
3. Anonymous, *Hepatitis C Fact Sheet No. 164*. 2000, World Health Organization.

13. SYMBOLS


Use-by date

Batch Code

Catalogue number


Temperature limit


Caution


Manufacturer

In Vitro Diagnostic Medical Device

14. ORDERING INFORMATION

Catalog Number: ABHCQ1 (25 rxn/box)
ABHCQ2 (50 rxn/box)
ABHCQ3 (100 rxn/box)

15. CONTACT INFORMATION

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