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Automated DNA Quantification using the dsDNA Quant-iT High Sensitivity Assay Kit and VICTOR³V/VICTOR X3

I. Purpose

This protocol outlines the procedure for quantification of DNA using the Quant-iT dsDNA high sensitivity assay kit and the VICTOR³V/VICTOR X3.

II. Scope

All procedures are applicable to the Library Core and Sequencing groups.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a Quality Systems associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Systems to audit this procedure for compliance and maintain control of this procedure.

V. References

Document Title	Document Number
Microlab Nimbus Manual (Version C)	IM.0302
Quant-iT dsDNA Assay Kit High Sensitivity User Manual	AM.0010
Wallac 1420 – Victor 3 Multilabel Counter Instrument Manual	IM.0067
2030 Victor X Multilabel Reader Instrument Manual	IM.0385

VI. Related Documents

Document Title	Document Number
Quant-iT HS Assay Analyzer Template	LIB_Forms.0016

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VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the safety data sheet (SDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number	Model or Catalogue #
VICTOR ³ V/VICTOR X3	Perkin Elmer	1420-040	✓
Micro-centrifuge	VWR	CABK 68826	✓
Eppendorf Centrifuge	Eppendorf	5810R	✓
Vortex Genie	Fisher Scientific	12-812	✓
Microlab Nimbus System	Hamilton	Nimbus	✓
Gilson P200 pipetman	Mandel	GF-23601	✓
P200 DF200 tips	Mandel	GF-F171503	✓
20µL Rainin multi-channel pipette	Rainin	L12-20	✓
20µL Rainin multi-channel tips	Rainin	RT-L10F	✓
OptiPlate-384 Black	Perkin Elmer	6007270	✓
96-well AB1000 plate	VWR	AB1000	✓
Plate, 12-column reservoirs	Seahorse	EK2057	✓
Quant-iT dsDNA HS Assay Kit 500 assays	Invitrogen	Q33120	✓
NIMBUS P50 tips 50µL Clear Sterile Tips, 5760 tips/case	Hamilton Co.	235831	✓
50mL Falcon Tube	Fisher Scientific	T406-2A	✓
Aluminum 3M Foil Tape	General Fasteners	34000740	✓
Bioshake XP	Q Instruments	001892	✓

IX. Procedure

1. Protocol Overview

This protocol utilizes the Hamilton Microlab NIMBUS system to help perform the following:

1. Dispense Quant-iT working solution into a 384-well Opti-Plate.
2. Transfer sample DNA and standards into a 384-well Opti-Plate.

Once setup on the liquid handler is complete, this protocol further describes the quantification and analysis of the samples and standards:

3. Measure sample fluorescence using the VICTOR³V/VICTOR X3 spectrophotometer.
4. Utilize the “Quant-iT Data Analysis Template” to calculate DNA concentrations from the raw fluorescence unit output from the VICTOR³V/VICTOR X3.

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For each set of VICTOR3V/VICTOR X3 measurements performed, a set of lambda DNA standards is used to generate a standard curve. These standards must be run in parallel with the samples and must be measured using the same Quant-iT working solution prepared for the sample DNA. The DNA standards are first aliquoted into a 96-well plate prior to being transferred to the 384-well plate. Each well contains eight replicates of the lambda dsDNA HS standards. Each 384-well plate will be aliquoted with DNA standards into Quadrant D and up to 3 x 96-well plates of sample into Quadrants A, B and C. The Quant-iT High Sensitivity Assay in 384-well format is scaled down by five times to achieve a 40µL total volume. Also, double the amount of dye (1:100 instead of 1:200) is added to extend the linear range. Therefore, this protocol is linear from 0-20ng/µL, or 0-40ng.

2. Set Up

- 2.1. Put on a clean pair of gloves and a lab coat. Wipe down the bench surface, pipettors, and small equipment with DNA Away using a large Kimwipe.
- 2.2. Remove the Quant-iT buffer, Lambda standards, and dye (if not already at room temperature) from 4°C and place them on the bench. 20-22mL Quant-iT buffer, 96µL each of the 8 lambda standards, and 200-220µL SYBR Green dye are required. Allow each to equilibrate to room temperature (it is crucial to let the dye thaw and equilibrate completely or inconsistencies in dye concentration may be observed). The Quant-iT dye is light sensitive and must be protected from light.
- 2.3. Retrieve the previously prepared sample DNA plate(s) and place on the bench to equilibrate to room temperature.
- 2.4. Once the dye has thawed and equilibrated to room temperature, mix the dye by vortexing gently and quick spin using the microfuge. Once the sample DNA plate has thawed, quick spin the plate(s) in an Eppendorf centrifuge on Program 1 (4°C for 1 minute at 2000g/1970rpm/700rcf).
- 2.5. Once the standards have equilibrated to room temperature, gently vortex or finger mix each standard and then quick spin using the microfuge. Go to Step 3 to manually prepare the standard plate or to Step 4 to prepare the standard plate using the Nimbus.

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3. Manual Preparation of Standard Plate

Obtain an AB1000 plate and label it “Standard Plate”. Aliquot the 8 replicates of 12µL of lambda standards into each row of the plate. Refer to the well map below for plate setup. This can be done by transferring 96µL of each concentration to column 1, and then using the Nimbus protocol "Quant-iT" to distribute the standards or a multi-channel pipettor to manually transfer 12µL of each concentration from column 1 to the remaining columns up to column 8.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0				
B	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5				
C	1	1	1	1	1	1	1	1				
D	2	2	2	2	2	2	2	2				
E	4	4	4	4	4	4	4	4				
F	6	6	6	6	6	6	6	6				
G	8	8	8	8	8	8	8	8				
H	10	10	10	10	10	10	10	10				

Table 1 – Manual standard plate setup

3.1. Proceed to Step 5 for preparing the Quant-iT working solution.

4. Automated Preparation of Standard Plate

4.1. Obtain an AB1000 plate and label it “Standard Plate”. Aliquot 96µL of lambda standards into column 1 of the plate. Refer to the well map below for plate setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0											
B	0.5											
C	1											
D	2											
E	4											
F	6											
G	8											
H	10											

Table 2 – Automated standard plate setup

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- 4.2. Log onto the Nimbus and select protocol "Quant-iT" or run protocol "LibraryConstruction Scheduler" and select the library type that is being processed. Select the "Quant-iT" step to run the Quant-iT protocol.

Note the "Quant-iT Setup" Program will have a version number (v) associated to it and will be present when running the protocol. All version numbers for Nimbus protocols have been removed on this document to streamline editing. Confirm on checklists or with a supervisor that the correct program is chosen.

NIMBUS: File> Production> Quant-iT > Quant-iT.med

The protocol will first ask the user "Do you need to distribute standards using the Nimbus?" If the standards were already manually distributed, select the "NO" radio button. Otherwise, select the "YES" radio and the Nimbus will aliquot 12µL of each concentration from column 1 across 8 columns of the same AB-1000 "standards" plate.

- 4.3. Quick spin the standards plate in an Eppendorf centrifuge on Program 4 (20°C for 1 minute at 2000g/1970rpm/700rcf). Set up Quant-iT working solution for next step on Nimbus.

5. Prepare Quant-iT Working Solution

- 5.1. Always wear the required personal protective equipment (lab coat, gloves, and safety goggles) when handling the Quant-iT reagent and working solution. Dispose of all Quant-iT solid/liquid waste in the SYBR Green disposal buckets.

- 5.1.1. For measuring 1-2 sample DNA plates:

In a 50mL Falcon tube, prepare 20mL of a 1:100 Quant-IT dye in Quant-IT buffer.

Quant-IT Buffer (Component B) = 20mL. If pre-aliquots are not available, use a Pipet Aid with a 25mL serological pipette.

Quant-IT Reagent (Component A) = 200µL

- 5.1.2. For quantifying 3 DNA plates:

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In a 50mL Falcon tube, prepare 22mL of a 1:100 Quant-IT dye in Quant-IT buffer.

Quant-IT Buffer (Component B) = 22mL. If pre-aliquots are not available, use a Pipet Aid with a 25mL serological pipette.

Quant-IT Reagent (Component A) = 220µL

5.2. Mix using the vortexer.

5.3. Keep the solution protected from light until ready for use. After dispense, ensure that the dye solution is evenly distributed across and within the columns of the reservoir. Remove any bubbles at the bottom of the reservoir with a clean pipette tip.

5.3.1. For 1-2 plate assays, dispense 1670µL of the dye solution into each column of the 12- column reservoir.

5.3.2. For 3 plate reactions, dispense volume should be 1840µL per column.

(Note: the product manual states the solution is stable for ~3 hours.)

6. Reaction Set Up on Nimbus

6.1. While still logged on from previous standard plate setup on Nimbus step, continue with second half of Quant-iT setup program:

NIMBUS: File> Production> Quant-iT > Quant-iT.med

6.2. Choose the appropriate number of 96-well sample plates to process. Set up the deck and confirm the deck layout on the screen before continuing the protocol. Document the location of each source plate and the corresponding quadrant.

6.3. The Nimbus program will transfer 38µL of working solution (Quant-iT Buffer + Dye) to each well of the selected quadrant(s) on the 384-well plate, changing tips for each transfer to match each sample and standards plate. It will then transfer 2µL of samples into the selected quadrant(s) with the same column sequence as their source 96-well sample plate(s) and finally 4µL of standards into Quadrant D. Be on-hand to quick-spin plates to remove bubbles and replenish the Nimbus P50 tips when prompted to do so for each DNA sample plate.

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- 6.3.1. Ensure that all 384 wells of the OptiPlate have been dispensed. Manually add 38µL of dye solution where necessary in case of any rare missed wells
- 6.4. When the program has finished, remove the 384-well plate from the deck and seal with 3M foil tape to block the light. Tightly seal the wells and the edges of the plate.
- 6.5. Vortex the plate thoroughly on the Bioshake XP using program 1 (2600rpm, 1 minute) and quick spin the plate in an Eppendorf centrifuge on Program 4 (20°C for 1 minute at 2000g/1970rpm/700rcf).
- 6.6. Incubate the plate at room temperature **in the dark** for 10 minutes to allow full intercalation between the dye and DNA. This incubation time is necessary to achieve the best linearity.
- 6.7. While the plate is incubating, remove the 96-well sample plate(s) from the deck and seal tightly with 3M foil tape and place on ice. Remove the Standard plate and discard into the garbage. Discard all Nimbus tips in the solid SYBR Green waste bucket. Dispose of the reservoir containing the Quant-iT working solution appropriately in the SYBR Green waste area.
- 6.8. Remove any remaining labware and tip holders from the deck. Clean up any spills.
- 6.9. Close the Nimbus software and log off the desktop.
- 7. Measure Fluorescence Using The VICTOR³V/VICTOR X3 Spectrophotometer**
 - 7.1. Log in to the VICTOR³V/VICTOR X3 computer with your user name and password. Double click on the “Wallac 1420 Workstation” icon. If using the VICTOR X3, open the PerkinElmer 2030 Workstation software and select ‘ignore’ to both ‘LID IS OPEN’ and ‘FRONT LID IS OPEN OR THE LID FOR THE EMISSION FILTER IS OPEN’.
 - 7.2. Under the “Tools” drop down menu select “Start Wizard” and click “Next”.
 - 7.3. In the “Protocols” folder select the program “Quant-iT HS OptiPlate-384F” and click “Next”. For VICTOR X3 “Quant-iT HS OptiPlate-384F” is located in the GSC folder.

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- 7.4. Carefully remove the 3M foil seal from the plate and place the plate on the spectrophotometer deck being careful not to contact the mirror. If the mirror is dirty, wipe it GENTLY with a damp Kimwipe. Close the lid and click “Next”.
- 7.5. List library ID and project name by quadrant in the comment section and click “Next”.
- 7.6. The spectrophotometer will measure the fluorescence readings. You can view the progress of the plate by clicking the “Live Display” tab (Figure 1). VICTOR X3 will automatically show the live display view.

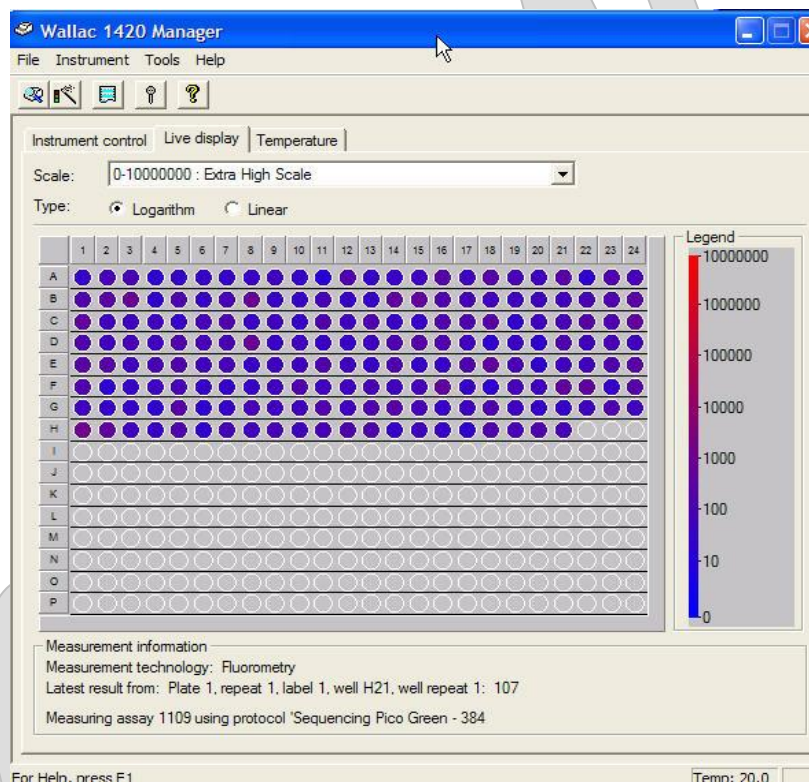


Figure 1 - Live display screen

- 7.7. Once the assay is completed, remove the 384-well plate and reseal with 3M foil tape.
- 7.8. Under the Tools drop down menu select “Results of Latest Run”.
- 7.9. Select “Export” under File and name the file. Include the name of the plate(s) processed, stage of library construction, the date and your initials. For example:

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tra23500_tra24560_tra23600_stock_110526nd

The Tray ID's must correspond to the source plate ID's if doing an initial QC quantification or the aliquot plate ID if doing QC quantification in the middle of library construction.

Save the file in the designated location in 'Library Core' folder (e.g. R:\Library Core\QC\Quant-iT). Go to that folder location and open the file to check that the data was successfully exported and the file exists.

- 7.10. Log off from the VICTOR3V/VICTOR X3 workstation and turn off the monitor. When shutting down the software for VICTOR X3, a PerkinElmer 2030 Manager window will ask 'Stop Instrument Server also?'. Select 'Yes' as this is a redundant software shut down window. Dispose plate from reader into the SYBR Green waste bucket.

8. Data Analysis

- 8.1. Open Analysis template named "Quant-iT HS Assay Analyzer Template" (LIB_Forms.0016) from R:\Library Core\QC\Quant-iT\Template. **You must save it as a new file or the data will be lost as well as the formulas.** Name that file with the same name as the raw data file adding the word "analysis" just before the date and your initials. For example:

tra23500_tra24560_tra23600_stock_analysis_110526nd

- 8.2. Save that file in a designated location in "Library Core" folder (e.g. R:\Library Core\QC\Quant-iT). You should have two files for each 384-well plate: a raw data file, and the analysis file.
- 8.3. Open the raw data file generated by VICTOR³V/ VICTOR X3. Copy and paste the data excluding column headings into sheet 1 of the **newly saved** analysis template in the 'Analysis' file. **Do not use the original template – Make sure that the name of the file displayed on the top of your screen is that newly saved file.**

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Plate	Repeat	Well	Type	Time	PicoGreen
1	1	A1	M	00:00:14.55	31
2	1	A02	M	00:00:14.92	125
3	1	A03	M	00:00:15.29	117
4	1	A04	M	00:00:15.66	45
5	1	A05	M	00:00:16.03	45
6	1	A06	M	00:00:16.40	48
7	1	A07	M	00:00:16.77	48
8	1	A08	M	00:00:17.14	36
9	1	A09	M	00:00:17.51	63
10	1	A10	M	00:00:17.88	36
11	1	A11	M	00:00:18.25	16
12	1	A12	M	00:00:18.62	156
13	1	A13	M	00:00:18.99	41
14	1	A14	M	00:00:19.36	40
15	1	A15	M	00:00:19.73	53
16	1	A16	M	00:00:20.10	242
17	1	A17	M	00:00:20.47	70
18	1	A18	M	00:00:20.84	368
19	1	A19	M	00:00:21.21	114
20	1	A20	M	00:00:21.58	78
21	1	A21	M	00:00:21.95	242
22	1	A22	M	00:00:22.32	21
23	1	A23	M	00:00:22.69	162
24	1	A24	M	00:00:23.06	100
25	1	B01	M	00:00:25.84	150
26	1	B02	M	00:00:26.20	260
27	1	B03	M	00:00:26.57	752
28	1	B04	M	00:00:26.94	36
29	1	B05	M	00:00:27.31	41
30	1	B06	M	00:00:27.68	41

Figure 2 - Raw data file generated by VICTOR³V

- 8.4. Once the data is pasted, the standard curve will be automatically generated. **Ensure the R² value of the standard curve is >0.95.** If this value is <0.95, the assay must be repeated and if <0.95 value is obtained again a new set of DNA standards must be prepared.

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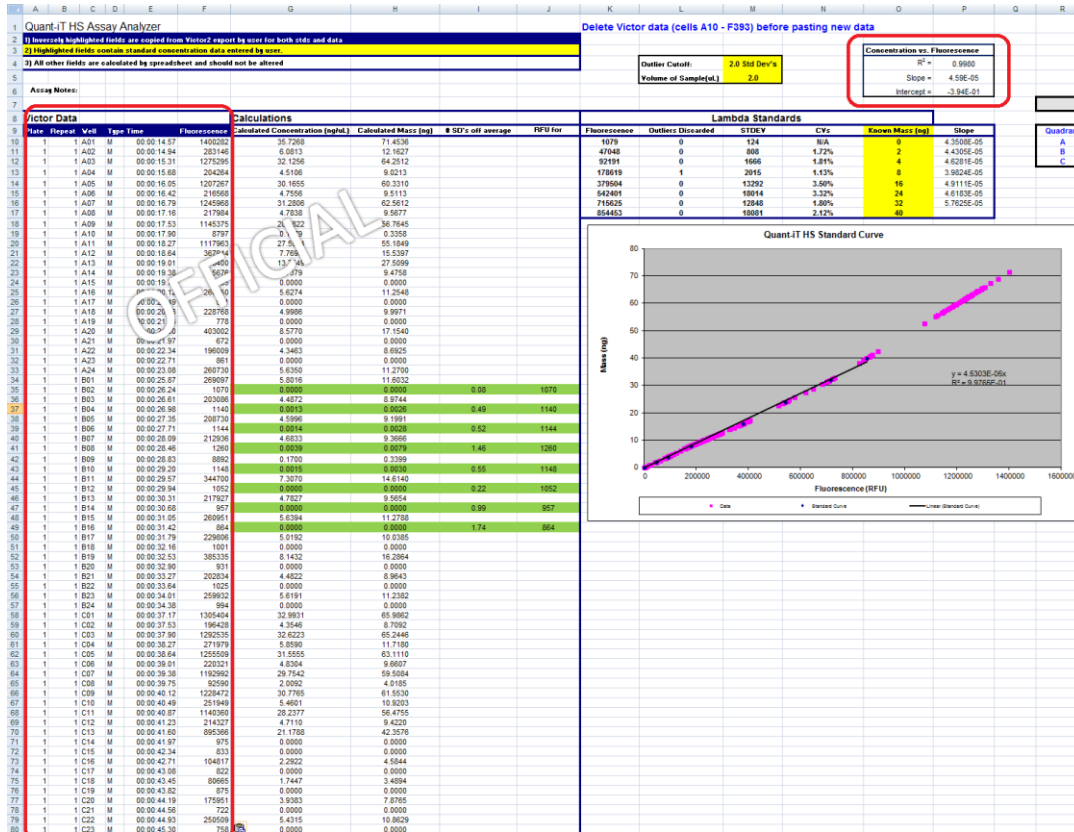


Figure 3 - Sheet 1 of LIB_Forms.0016

- 8.5. Blue dots correspond to DNA standards and pink dots correspond to the measured values. Make sure that your measured values lie within the region of the standard curve (0-40ng).
 - 8.6. The concentration of the DNA samples will be calculated in column G on the spreadsheet.
- Note: It is important that no cells are shifted in any direction on the spreadsheet or the template will not work.**
- 8.7. The calculated concentrations will automatically be imported into the “sorting” sheet.
 - 8.8. Once sorting is complete, the data sorted into each quadrant is automatically imported into the corresponding sheets labeled “Quad A”, “Quad B” and “Quad C”. You may

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change the names of the sheets by right clicking each tab and selecting “rename”. The tab names can be changed to the Library IDs.

- 8.9. In each of sheets Quad A, B and C, type in the plate ID and the File name at the top of the sheet. Also record which wells are the plate control wells by highlighting these cells in blue.
- 8.10. Additional analysis needs to be done for each of Quad A, B and C. In “**Quad A**” sheet, column B contains the calculated concentrations of each well. Concentrations that are highlighted in yellow are out of range of the Quant-iT standard curve. Quant-iT quant must be repeated with appropriate dilutions if the number of highlighted sample wells is greater than 10% of the total number of sample wells.
- 8.11. Column C allows you to manually enter the calculated concentrations of any samples in which the Quant-iT quant was repeated. The cell which contains values out of range of the standard curve will be highlighted in green.
- 8.12. The actual concentration of each sample needs to be calculated by incorporating the dilution factor. In column E, select which values you would like to use to calculate the final concentration. For example in cell E03, you may type = **A03**. This will allow you to use the value in cell A03 to calculate the final concentration. Alternatively, “=B03” can be typed into cell E03 if a Quant-iT quant was redone. All values in column E must be in the range of the standard curve.
- 8.13. Type in the dilution factor used in the column F under “dilution factor”.
- 8.14. Row G will contain the values for the actual concentration.
- 8.15. In column H, use the excel formula “=average(cell range)” to calculate the average of the samples. Do not include positive/negative controls that are present on the plate in this calculation since their concentrations may not be representative of the rest of the working samples on the plate.
- 8.16. Type in the sample volume of each sample in column I.
- 8.17. Row H has automatically calculated the total amount of DNA in ng for each sample.
- 8.18. Repeat steps 8.10-8.17 for Quad B and Quad C sheets.

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8.19. Save the analyzed version and consult your supervisor before proceeding to the next step.

8.20. If the quant has been performed on stock DNA plates, run the "96well_Quant-iT_QC" protocol in LIMS.

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