Technical note

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Custom Method for colorimetric quantification of cellular cytotoxicity

NanoDrop Spectrophotometers

Lactate dehydrogenase (LDH) is a cellular cytotoxicity marker that is released into the cell medium when the integrity of the plasma membrane is diminished.[1] Quantifying released cytosolic LDH is useful for many applications such as monitoring the efficacy of cancer therapeutics, evaluating the cytotoxicity of drugs in development, performing quality control of cell lines in manufacturing facilities, and more.[2-4]

The released LDH is quantified through the enzymatic reaction outlined in Figure 1. LDH catalyzes the conversion of lactate to pyruvate by reducing NAD+ to NADH; diaphorase then uses NADH to reduce tetrazolium salt (INT) to a red formazan product.[5,6] This enzymatic reaction provides a method for colorimetric quantification at 490 nm using a Thermo Scientific™ NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer. For higher-throughput applications, the Thermo Scientific™NanoDrop™ Eight 8-channel Microvolume UV-Vis Spectrophotometer can help speed-up sample processing.

Experimental procedures

NanoDrop Custom Method

A Custom Method was created on the NanoDrop One/One^{C} PC Control Software to quantify the red formazan product, which is directly proportional to the amount of extracellular LDH. [Note: The Custom Method file can be downloaded from the software section of the NanoDrop homepage at thermofisher.com/nanodrop. A Custom Method was also created for the NanoDrop Eight instrument to support high-throughput laboratories and it too is available on the website.] The method utilizes light in the visible range (350-850 nm) with an analysis wavelength of 490 nm and baseline correction set to 680 nm to eliminate the requirement of correction calculations outside of the software.

The User Factor is 1 Unit, which ensures the software reports the absorbance without any additional calculations in Beer's Law. The method also includes the selection of Automated Pathlength to ensure the best pathlength is utilized depending on the absorbance intensity at the analysis wavelength to prevent detector saturation. All prepared samples were measured in 2.0 µL volumes using the microvolume pedestal on the NanoDrop One instrument.

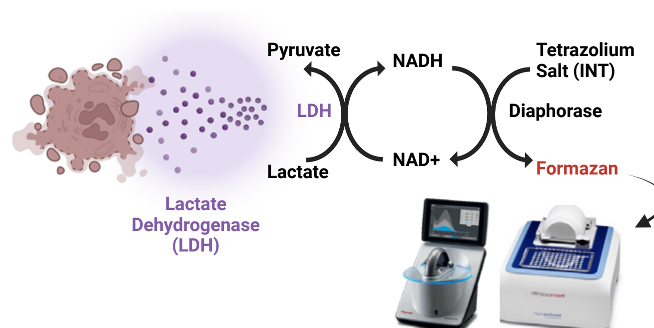

Figure 1. Graphic representation of the LDH enzymatic reaction starting with the release of LDH from a damaged cell and finishing with the red formazan product that can be quantified with the NanoDrop One Spectrophotometer or the NanoDrop Eight Spectrophotometer. Figure created with BioRender.com.

CyQUANT LDH Cytotoxicity Assay

The Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen, C20301) is supplied with the necessary reagents to perform the colorimetric assay. To determine the linear range of the assay and the optimal dilution, the assay was conducted per manufacturer's instructions with the 1X LDH Positive Control in lieu of a mammalian cell culture. The 1X LDH Positive Control was serially diluted 1:1 from 10 mg/mL to 0.625 mg/mL of BSA (Table 1) and served as the Maximum LDH Release samples.

Sample	Concentration (BSA)
1	0.625 mg/mL
2	1.25 mg/mL
3	2.5 mg/mL
4	5 mg/mL
5	10 mg/mL

Table 1: Dilutions of BSA used to determine the linear range of the LDH Cytotoxicity Kit.

The Spontaneous LDH Release samples used the same 1:1 serial dilutions of BSA but did not include the LDH Positive Control. The Maximum LDH Release samples used 10X Lysis Buffer while the Spontaneous LDH Release samples used water. A spontaneous control provides information on whether media with serum contains LDH that may artificially inflate the amount of LDH released from the cells. The solution used to blank the NanoDrop instrument included 50 µL of each of the following components: 10X Lysis Buffer, Reaction Mixture, Stop Solution, and culture media. Preparing the blank solution in this manner maintains the same concentrations of each component used for the samples to ensure the NanoDrop instrument was blanked properly.

Results

The spectrum of the red formazan product used to quantify the amount of extracellular LDH is shown in Figure 2, with the absorption maximum at 490 nm. The absorbance measurements at 490 nm of all five samples for the Maximum and Spontaneous LDH Release are outlined in Table 2. Samples were measured in triplicate and the averages and standard deviations of each sample were calculated.

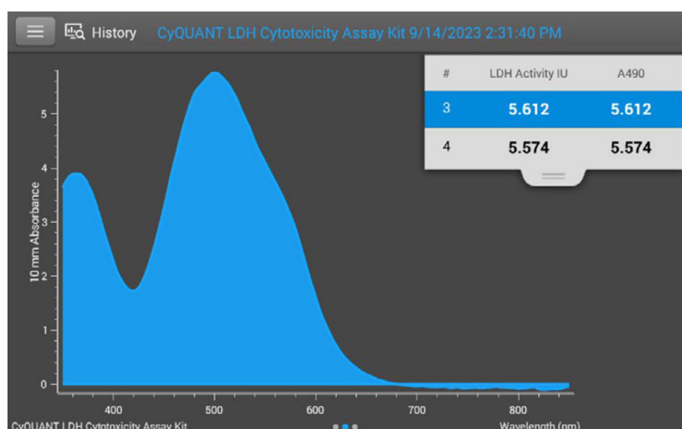

Figure 2. Screenshot of the NanoDrop One Local Control software displaying the spectrum of the red formazan product.

BSA Concentration (mg/mL)	Maximum LDH Release (Abs)	Standard Deviation -Max. (mg/mL)	Spontaneous LDH Release (Abs)	Standard Deviation-Spon. (mg/mL)
0.625	1.51	0.05	0.019	0.006
1.25	2.6	0.2	0.01	0.01
2.5	4.35	0.05	0.02	0.01
5	5.79	0.01	0.015	0.006
10	6.473	0.009	0.002	0.009

Table 2. Average absorbance measurements and standard deviations calculated from triplicate measurements using the LDH Cytotoxicity Assay Custom Method on a NanoDrop One Spectrophotometer.

The Spontaneous LDH Release values were close to zero, indicating a low amount of serum-associated LDH. The standard deviations for Maximum LDH Release samples range from 0.009 to 0.154 mg/mL and 0.006 to 0.014 mg/mL for the Spontaneous LDH Release samples. The low standard deviations for each sample display high sample-to-sample reproducibility with the NanoDrop One Spectrophotometer.

The average absorbances at 490 nm were plotted in Figure 3. Instrument and assay linearity were determined to occur from Sample 1 to Sample 3, indicating the optimal cell number for the assay lies within this linear range. The optimal cell number can then be used for subsequent experiments to investigate chemical and drug mediated cytotoxicity using the same NanoDrop Custom Method and LDH Cytotoxicity Assay Kit. First determining the optimal cell number is crucial for successful cytotoxicity experiments, to ensure the cell number falls within the assay and instrument linear range.

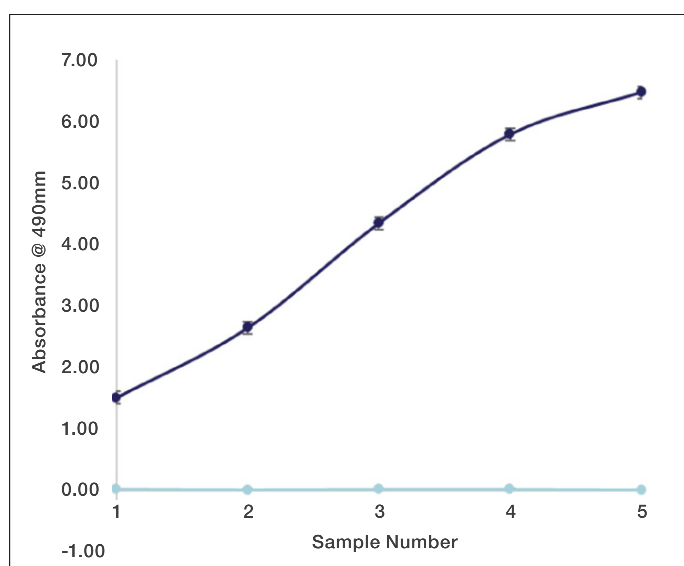

Figure 3. Absorbance at 490 nm measured with the NanoDrop One Spectrophotometer graphed against sample number. Maximum LDH Release is plotted in dark blue and Spontaneous LDH Release is plotted in light blue. Error presented as mean ± SD.

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Conclusions

Investigating cellular cytotoxicity is a common requirement in cancer therapeutics, drug development, and manufacturing QA/QC. The NanoDrop One/One^{C} Spectrophotometer, the NanoDrop Eight Spectrophotometer, and their associated Custom Method provide a reliable and reproducible method of measuring released LDH as a function of cellular cytotoxicity. The NanoDrop Eight Spectrophotometer can measure the absorbance of eight samples simultaneously, providing a reduction in time spent at the bench for high-throughput laboratories testing cytotoxicity. Having a simple colorimetric assay and a trusted spectrophotometer such as those presented here can kickstart high-quality cytotoxicity experiments.

References

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