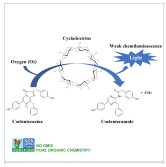

Version 3

Jul 12, 2021

Chemiluminescence of coelenterazine catalyzed by cyclodextrins as a luminescence reference standard for luminometers V.3

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Misha Koksharov

ABSTRACT

Bioluminescence and chemiluminescence are widely used in sensitive detection methods in biomedical sciences and analytical chemistry. A limitation of this type of measurements is that luminometers and platereaders do not directly quantify absolute quantum output of the reaction but report "relative luminescence units" (RLU) which are specific for a given instrument and reaction vessel design. At the same time, there are no simple and convenient luminescence reference standards that would have been universally available, so results (RLU measurements) reported by different instruments and laboratories usually cannot be directly compared.

I have found that cyclodextrins - which are often used to solubilize coelenterazine (CTZ) analogs and other compounds in water buffers - catalyze a weak chemiluminescence of CTZ (and its analogs). Chemiluminescence of 20 μM CTZ in the presence of 10 mM β-cyclodextrin or 10 mM trimethyl-β-cyclodextrin in the 50 mM Na-phosphate buffer (pH 7.40) can be used as a simple and convenient reference standard to define and compare RLU readings obtained by different instruments. This system is composed of only small molecules of a defined chemical composition which are not expensive and available in high purity from multiple suppliers making this system convenient for the general use as a luminescence reference standard.

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KEYWORDS

chemiluminescence, bioluminescence, luminescence, reference standard, coelenterazine, cyclodextrins, luminometer, luminescence platereader, beta-cyclodextrin, trimethyl-beta-cyclodextrin, luciferase

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Jul 12, 2021

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51482

GUIDELINES

CHEMICAL BASIS OF THE METHOD

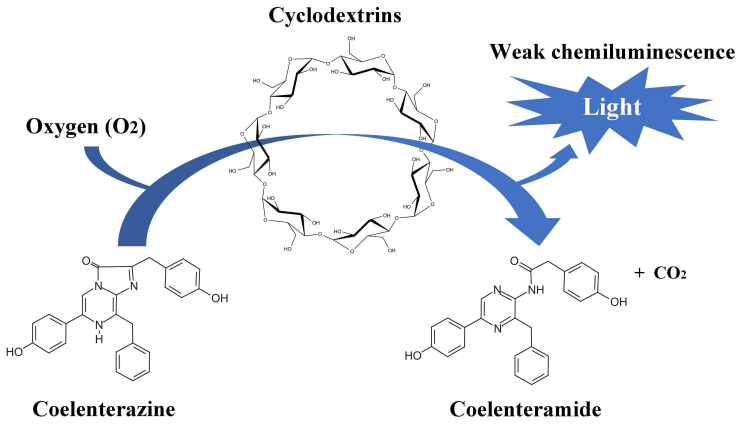

Fig. 1. A scheme illustrating the light emitting reaction of coelenterazine (CTZ) in the presence of cyclodextrins (CDs).

The method relies on the ability of cyclodextrins to catalyze light emission from coelenterazine (CTZ) in water buffers (Fig. 1). Coelenterazine itself is a high-energy compound which has the ability to emit chemiluminescence but it is very low under normal conditions (Fig. 2) and hard to reliably detect above the background on many instruments. This uncatalyzed luminescence appears to result mainly from oxidation by reactive oxygen species and to a less extent by molecular oxygen (Lucas & Solano, 1992; Teranishi & Shimomura, 1997a; Teranishi, 2006). In the presence of cyclodextrins, light emission can be enhanced to various degrees, up to more than 1000-fold (see **Note 1**). This is weak compared to catalysis by CTZ-dependent luciferases but sufficient for reliable detection on most instruments.

In the current protocol this phenomenon was used to make a convenient reference standard for luminescence to calibrate values obtained on different instruments. This standard uses 10 mM TMCD or 10 mM β-CD (the first one leads to about 20-fold higher luminescence than the second one) to produce chemiluminescence from 20 µM CTZ in 50 mM Na-phosphate buffer (pH 7.40) (Fig. 3). All the components of these mixtures are small molecules of a defined composition. They are available from multiple suppliers in high purity and are easy to use and store. CTZ is a substrate of many widely used luciferases, so it is routinely present in many labs using bioluminescent assays. Therefore, the described assay mix could be easily reproduced giving a similar light output to use a reference.

This addresses the common limitation of luminescence measurements which report relative luminescence units (RLU) specific for a given instrument. There are a few possible reference standards based on chemiluminescence of luminol or on radioactivity (Lee & O'Kane, 2000) but they did not received a wide adoption due to being technically challenging. Since there are no convenient reference standards, results (RLU values) obtained on different instruments and reported by different research groups usually cannot be directly compared. For example, when measuring specific activity of purified luciferase enzymes or light emission from luciferase reporters in live cell cultures, it is usually not possible to determine how the measured absolute values compare to the values reported in previously published studies or made previously on another instrument.

A practical example is values of weak chemiluminescence of CTZ analogs in PBS buffer in the presence of 0.5 mg/ml bovine serum albumin (BSA). This is a less repeatable reference system: up to 2-3 fold difference can be allowed between experiments conducted in different locations due to different lots of BSA and uncertainties in CTZ analog concentrations. This system leads to about 80 RLU/sec (10µM furimazine) and 170 RLU/sec (10 µM coelenterazine-h) when measured on the Biotek HTX plate reader (Biotek) used in the current protocol (default settings: PMT gain 135; BSA is cat #A-420-10 from GoldBio) while the values reported in the literature (see Fig. S4B in Machleidt et al, 2015) for the GloMax Multi+ plate reader (Promega) are about 14000 and 30000 RLU/sec, respectively (175-fold higher).

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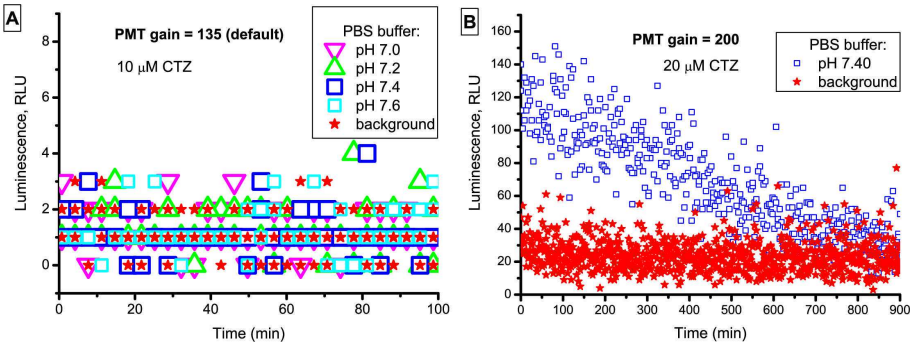

Fig. 2. Chemiluminescence of CTZ in the PBS buffer (pH 7.4) without catalysts added.
A) 10 μM CTZ: not detectable relative to the background luminescence level when using the default PMT gain = 135.
B) 20 μM CTZ: about 4 times higher than the background when using the PMT gain = 200.
Recordings were made using 150 μl reaction mix per well in the 96-well white opaque microplate.
PBS buffer: 8.2 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 148 mM NaCl, pH 7.4 (at RT).
Note: raising the PMT gain parameter from 135 to 200 increases the absolute signal about 20-fold but also increases the background noise.

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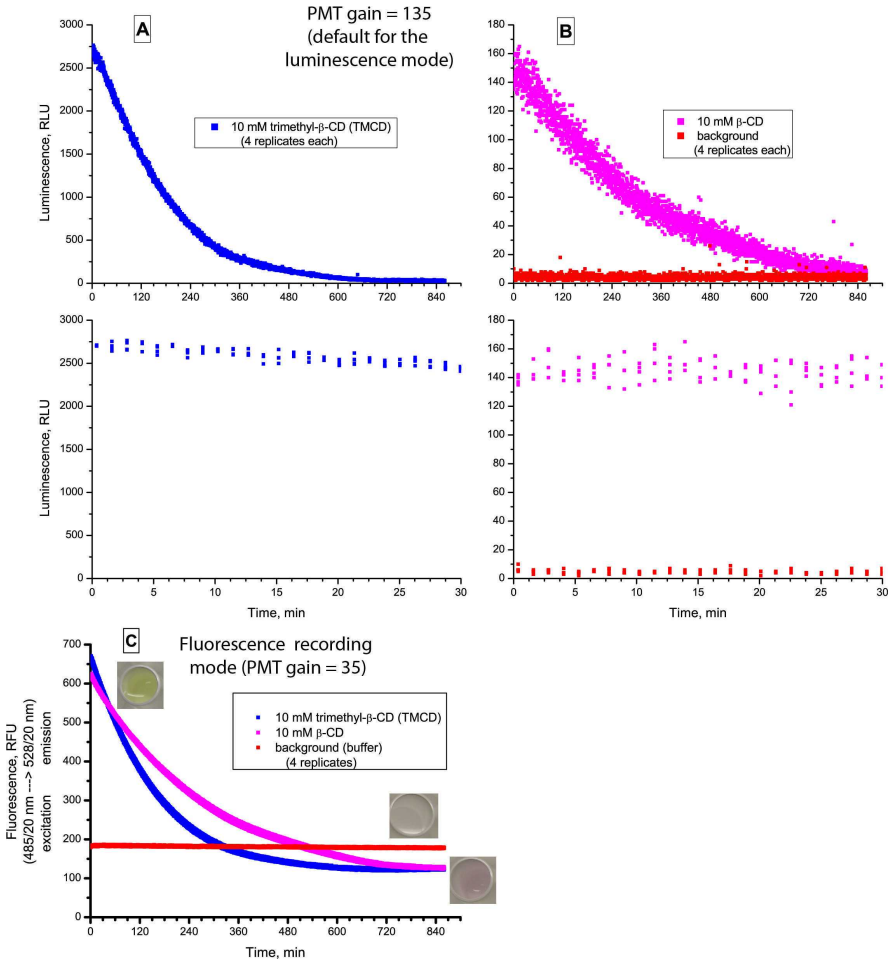

Fig. 3. Kinetics of chemiluminescence (A, B) and of degradation (C) of 20 µM CTZ in the presence of 10 mM TMCD and β-CD in the reference assay buffer (50 mM Na-phosphate, pH 7.40). The measurements were conducted in a non-transparent white 96-well plate (Perkin Elmer, USA), 100 µl reaction mix per well. Luminescence (RLU/sec) and fluorescence (RFU) values *were not* corrected for the background.
A) Luminescence decay in the presence of 10 mM TMCD.
B) Luminescence decay in the presence of 10 mM β-CD.
C) In parallel to luminescence measurements, the CTZ degradation in the presence of TMCD and β-CD was monitored by fluorescence of CTZ (bandpass filters: 485/20 nm excitation → 528/20 nm emission).
♦ At the late stages of CTZ decomposition in the presence of cyclodextrins, the fluorescence decreases below the background level of the blank buffer (panel "C") due to the formation of dehydrocoelenterazine-related products which partially absorb light in the emission and excitation band windows (see the representative well image inserts in the panel "C").
♦ The background fluorescence level is determined by the cross-talk between the emission and excitation filters and the degree of the resultant pass-through light reflection back into the fiberoptic probe.
♦ Luminescence is relatively stable (lower panels in "A" and "B") during the first 10 min (TM-β-CD) and 30 min (β-CD) of the reaction and can be used as a reference standard (Table 1).

How the signal varies depending on differences (i.e. uncertainties and errors) in the composition of the assay mix:

- 10 mM TMCD concentration is in the less than proportional interval of the "luminescence - [TMCD]" response curve, so small variations in TMCD (during preparation and due to possible variation of the water content) should not considerably affect the light output of the standard. For example, changing TMCD concentration by +5 mM or -5 mM from 10 mM leads to a +25% and -40% change in chemiluminescence (with 20 µM CTZ).
- 20 µM CTZ is in the semi-saturating range of the "luminescence - [CD]" curve for 10 mM β-CD and at the close-to-saturation range for 10 mM TMCD. For example: with 10 mM β-CD, changing CTZ concentration by +5 µM or -5 µM from 20 µM leads to a +18% and -15% change in chemiluminescence; with 10 mM TM-β-CD this causes a +5% and -20% change in chemiluminescence.
- Changing pH by -0.20 and +0.20 units from pH 7.40 results in approximately 17% decrease or 20% increase in chemiluminescence, respectively.

The described system as a reference standard when compared to alternatives.

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The described system as a reference standard when compared to alternatives:

The current system "CTZ + β-CD/TMCD in Na-phosphate buffer":

1) All components are chemically defined small molecules.
2) All components are readily available at high purity from many suppliers and easy to store long-term.
3) Chemiluminescence is relatively stable (10-30 min) for measurements under practical conditions.
4) Since no quantum yield data is available for now, this system can serve only as a relative standard but cannot be used to obtain absolute photons/sec values.

Alternatives and their limitations:

1) Luminol chemiluminescence is the classic absolute reference standard (Lee & O'Kane, 2000) but it is rarely used for referencing the absolute light output due to being technically challenging. This system exhibits a fast flash-type kinetics (problematic to use on instruments not supporting it), depends on concentrations of many components which may be harder to standardize (hydrogen peroxide, metal ions, etc) (Maezte et al, 2009). Very few papers implement some version of this standard (Suzuki et al, 2016; Loening et al, 2007). It would be convenient to have a reference to the CTZ/cyclodextrin reaction using this standard.
2) Luciferase enzymes - if used as a reference - are subject to considerable variations between different lots and suppliers as well as to degradation during shipping and storage; their activities are rarely standardized and they are expensive. A weak chemiluminescence in the presence of bovine serum albumin (BSA) belongs to this group.
3) Potentially, the glow-type chemiluminescene of CTZ and its analogs in some organic solvents can be considered (e.g. in DMSO, PEG-400, PPG-425, etc) but it is not very reproducible since it heavily depends on the presence of impurities such as water or trace bases.
4) A few publications utilize in-house made and standardized light-emitting diods (Yoshita et al, 2017; Wang et al, 2020) which are not available for a wide adoption and have a fixed geometry.

LITERATURE BACKGROUND AND SUPPORTING DATA

Coelenterazine and related compounds are poorly soluble in water-based buffers. In the PBS buffer (pH~7.4) the thermodynamic solubility of CTZ (i.e. in equilibrium with a crystalline precipitate) is below 10 µM, but supersaturated CTZ solutions up to about 50 µM can be obtained by a rapid dilution of concentrated stocks made in organic solvents.

Cyclodextrins - cyclic polymers of glucose - are often used to increase the *nominal* solubility of hydrophobic small molecules. Derivatives of α-CD, β-CD and γ-CD are usually used: they have a ring composed of 6, 7 or 8 glucose units, respectively, with a size of the inner cavity increasing in this row. They form a complex with CTZ by binding it in the hydrophobic cavity, and CTZ becomes solubilized as a part of such complex. β-CD derivatives are usually the most effective for CTZ. For example, about 4 mM CTZ can be dissolved in 100 mM (14% w/v) HP-β-cyclodextrin solution (Teranishi & Shimomura, 1997b). About 12 mM CTZ and 2 mM h-CTZ can be dissolved in 40% w/v (185 mM) SBE7-β-CD (Captisol) (M. Koksharov, *unpublished*) which is the least toxic cyclodextrin (Luke et al, 2010) for intravenous injections and serum-free cell culture. Methylated β-CD variants (DMCD, RMCD) appear to be the most efficient solubilizers of CTZ variants (M. Koksharov, *unpublished*) but they are toxic to mammalian cells even at very low concentrations due to the rapid extraction of cholesterol from cellular membranes (Szente & Fenyvesi, 2017; Szente et al, 2018).

When testing cyclodextrins as CTZ solubilizers, I have found that solutions of cyclodextrins catalyze the chemiluminescence of CTZ to a various extent depending on the type of cyclodextrin (Fig. 4). The enhancement is weak with the unsubstituted β-CD, higher with SBE7-β-CD or HB-β-CD, and the strongest with methylated β-CD derivatives. Since cyclodextrins bind CTZ in their hydrophobic cavity, this effect is consistent with previous observations that a number of proteins binding CTZ (e.g. serum albumin) can catalyze a weak chemiluminescence reaction (Viviani & Ohmiya, 2006; Vassel et al, 2012; Inouye & Sahara-Miura, 2014; Nishihara et al, 2020).

While some cyclodextrins (e.g. β-CD, HP-β-CD, SBE7-β-CD at 10 mM) do not affect or even stabilize the CTZ in solution relative to its typical half life of 3-4 hours at pH 7.4, a few cyclodextrins (>10 mM) and, particularly, serum albumin (> 4 mg/ml) and serum (>5-10% v/v) can considerably increase the rate of CTZ degradation. RMCD and

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TMCD reduce it to 2-2.5 h (Fig. 3); 4 mg/ml BSA or 10% FBS (fetal bovine serum) reduce it to 35-45 min (at room temperature), and in 100% serum (FBS) at the physiological 37°C temperature the half-life of CTZ can drop to just 8 minutes. In the latter cases the weak luminescence reflects a low quantum yield but the actual catalyzed CTZ degradation can be relatively fast which can affect cell culture and *in vivo* applications.

Several relevant findings were reported for vargulin - a substrate of Vargula/Cypridina luciferase - which has the same imidazopyrazinone core as CTZ. Its chemiluminescence was also shown to be enhanced by albumin and a few other binding proteins (Kanie et al, 2020). β-CD and its methylated derivatives increased the already relatively high chemiluminescence of MCLA (a vargulin analog), with 10 mM dimethyl-β-CD providing a 6-fold increase in luminescence (Mitani et al, 1995). Chemiluminescent oxidation of luminol by hydrogen peroxide catalyzed by metal ions could be enhanced up to 30-fold in the presence of 140 mM of several cyclodextrins (Maezte et al, 2009).

Commonly used cyclodextrin compounds have different types of substitutions introduced at OH groups of the glucose rings. These substitutions can be variable in terms of positions and number, so different lots and products of different manufacturers tend to vary between each other, sometimes leading to differences during applications (Schönbeck et al, 2011). Among the compounds shown in the Note 1, RMCD (methyl-β-CD, randomly methylated), HP-β-CD (hydroxypropyl-β-cyclodextrin) and SBE7-β-CD (sulfobutylether-β-cyclodextrin) have an average degrees of substitution of 11.9, 5.6 and 6.5, respectively, per a single CD molecule. The parent β-CD compound and the fully methylated (21 methyloxy groups per molecule) TM-β-CD are small molecules of a defined chemical composition, so they are convenient to use in a reference standard due to the low lot-to-lot variation in composition as long as they are of high purity.

Note 1. Chemiluminescence of CTZ catalyzed by different cyclodextrins.

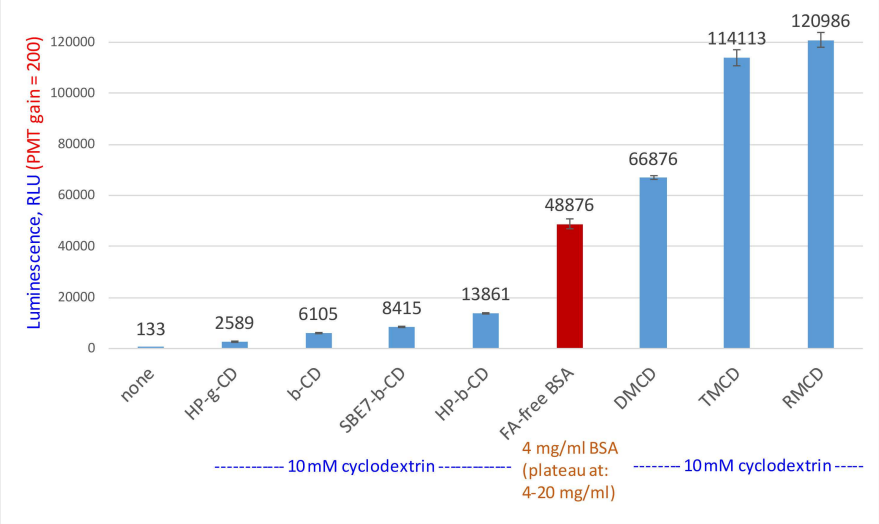

Fig. 4. Representative chemiluminescence of 20 µM CTZ in the presence of 10 mM of various cyclodextrins or 4 mg/ml bovine serum albumin (BSA, "fatty acid free") in the PBS buffer (pH 7.4). 240 µl per well. Error bars are standard deviations. PMT gain = 200.

Reagents were the following (catalog numbers): HP-γ-CD (d.s.=0.6; Sigma, #H125-5G-I), β-CD (d.s.=0; Acros, #AC406000250), SBE7-β-CD (d.s.=0.93; CyDex, #RC-0C7), HP-β-CD (d.s.=0.8; MP Bio, #0215354001), methylated β-CDs: RMCD (d.s.~1.7; Acros, #AC377110050), DMCD (d.s.=2; Sigma, #39915-1G), TMCD (d.s.=3; TCI, #T10941G). d.s. - degree substitution per a glucose ring (from 0 to 3).

"Fatty acid free" BSA (Goldbio, #A-421-10) was included for a comparison (this particular product had the highest activity among several other FA-free BSA products and about 2-fold higher activity compared to non-FA-free BSA products from different suppliers).

Randomly methylated cyclodextrin (RMCD) produces the highest chemiluminescence with CTZ - somewhat higher than TMCD - as well as with many other CTZ analogs (hCTZ, bisCTZ, Prolume Purple series). Since RMCD is relatively inexpensive, it can be potentially used in chemiluminescence assays based on CTZ analogs having brighter starting autoluminescence (Mitani et al, 1995) and to analyze spectral emission properties of various new synthetic CTZ analogs in "enzyme-like" conditions. Many of them do not work with any available luciferases (Gagnot et al, 2021) but could still hold promising properties which can be evaluated in the

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cyclodextrin system, and then new luciferases can be evolved that would accept these substrates.

ACKNOWLEDGEMENTS

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MATERIALS TEXT

You will need the following reagents, solutions and equipment.

EQUIPMENT

1) Luminometer, luminescence plate reader, luminescence imager - for which you would like to obtain the reference RLU values.

In this protocol the RLU values of the Synergy HTX plate reader (Biotek, USA) that I'm currently using were measured as an example. This instrument has a complex optical system (fiber-optic cables, filters, empty space intervals) between the sample and the photomultiplier tube (PMT) detector leading to considerable losses of light. Due to these losses (and a more red-light-sensitive PMT unit), it is likely one or two orders of magnitude less sensitive than the instruments where the PMT unit is located close to the sample. For example, such as TopCount NXT luminescence plate reader (Perkin Elmer, USA) (Koksharov, 2021) and FB12 single-tube luminometer (Berthold, Germany) that I've worked with previously (but there are no reference values available to compare them directly). GloMax Multi+ plate reader (Promega) generates about 175-fold higher absolute RLU values according to the estimates based on literature data as mentioned in the Introduction.

2) Plates or tubes used in the assay: the RLU values depend on the reaction vessel type, vessel and reader geometry, use of lids (for top-reading instruments), etc.

In this protocol, the results for the following plates were obtained:

- 24-well CytoOne cell culture plates **USA Scientific Catalog #CC7682-7524** (bottom readings are identical to
- 24-well Costar cell culture plates **Corning Catalog #3524**)
- 96-well Nunc black clear bottom tissue-culture plates **Thermo Fisher Scientific Catalog #165305**
- OptiPlate-96 White Opaque 96-well Microplate **Perkin Elmer Catalog #6005290**

3) Spectrophotometer: to precisely quantify the concentration of CTZ in the stock solution.

In addition to classic quartz cuvettes, the disposable (but reusable) UV plastic cuvettes can be also convenient:

- BRAND UV-Transparent Disposable Cuvettes, Ultra-micro (15mm window height) **BRAND Catalog #759220**
- BRAND UV-Transparent Disposable Cuvettes Ultra-micro (8.5mm window height) **BRAND Catalog #759200**

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Cuvette Caps round for ultra-micro cuvettes **BRAND Catalog #759240**

Disposable polystyrene (PS) and polymethyl methacrylate (PMMA) cuvettes will also work fine for measurements above 400 nm. PMMA cuvettes generally cannot be reused after ethanolic solutions (they develop a pattern of micro-cracks if filled again with ethanol after drying).

SOLID CHEMICALS

CAS numbers and potential suppliers of reagents are listed.

1) Coelenterazine (CTZ).

Coelenterazine **Gold Biotechnology Catalog #CZ2.5** (used in this work)

Coelenterazine **NanoLight Technology Catalog #303-500** (used in this work)

Store the dry solids at -80°C, protected from light.

2) β-cyclodextrin (β-CD)

beta-Cyclodextrin, >98% **Acros Organics Catalog #406000250** (used in this work)

beta-Cyclodextrin **Sigma Aldrich Catalog #C4767**

3) trimethyl-β-cyclodextrin (TM-β-CD, TMCD, TRIMEB, Heptakis-(2,3,6-tri-O-methyl)-beta-Cyclodextrin)

Trimethyl-beta-cyclodextrin, >98% **TCI Chemicals Catalog #T10941G** (used in this work)

trimethyl-beta-cyclodextrin **Sigma Aldrich Catalog #51707**

Can be obtained cheaper in higher quantities from the Cyclodextrin-Shop:

trimethyl-beta-cyclodextrin **Cyclodextrin-Shop Catalog #CDexB-032**

Methyl-β-cyclodextrin (RMCD, RAMEB) is cheaper than TMCD and generally leads to a similar (slightly higher) light output but this randomly methylated compound can differ considerably lot to lot even for the same supplier (Schönbeck et al, 2011).

If relatively frequent routine reference measurements are desired, it can be used instead of TMCD but any new lot must be initially referenced against TMCD/β-CD which have a defined and thus reproducible chemical composition.

Methyl-beta-cyclodextrin average Mw 1310 **Acros Organics Catalog #AC377110050**

(The lots from ACROS Organics™ usually have an average degree of substitution of 1.7 per glucose ring (by NMR) according to available Certificates of Analysis.)

The distributions of substitutions in different methylated β-CD derivatives are nicely illustrated in the Figure 1 of the following paper: (Varga et al, 2019).

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4) sodium phosphate monobasic (NaH_2PO_4)

Sodium phosphate monobasic monohydrate **Mallinckrodt Catalog #7892** (used in this work)

Sodium phosphate, monobasic, 99%, for analysis, anhydrous **Acros Organics Catalog #AC389872500**

SOLVENTS

1) propylene glycol (>99.5%)

propylene glycol **Sigma Aldrich Catalog #82280-250** (used in this work)

2) ethanol (absolute)

Ethanol absolute **PharmCo Catalog #111000200** (used in this work)

3) high purity water

For example, ultrapure water (resistivity>18.2 MΩ·cm) generated by the Milli-Q system (Millipore, USA).

PREPARATION OF BUFFERS AND STOCK SOLUTIONS

1.1x concentrated 50 mM Na-phosphate assay buffer (pH 7.40)

Prepare the 1.1x concentrated (55.5 mM Na-phosphate) assay buffer in water.

Adjust the pH to 7.40 with NaOH. pH **7.40** **Room temperature** **21-22 °C**

A precise determination of pH and the use of a pH reference standard in essential, because the CTZ chemiluminescence depends on pH.

Filter and store at RT. **Room temperature**

10 mM trimethyl-β-cyclodextrin in 1x assay buffer (50 mM Na-phosphate, pH 7.40)

Weight 572 mg of TMCD (M= 1429.54; **adjust the amount** for the water content if available (e.g. from a Certificate of Analysis which can be presented as a "loss on drying" or "by Karl Fischer") per 40 ml final solution.
Put into a 50 ml Falcon-type tube, add 36 ml 1.1x buffer, adjust the volume to 40 ml. Dissolve 10 mM TM-β-CD by mixing or vortexing.
Store frozen for the long-term.

10 mM β-cyclodextrin in 1x assay buffer (50 mM Na-phosphate, pH 7.40)

Weight 454 mg of β-CD (M=1134.98; **adjust the amount** for the water content if available (e.g. from a Certificate of Analysis which can be presented as a "loss on drying" or "by Karl Fischer") per 40 ml final solution.
Put into a 50 ml Falcon-type tube, add 36 ml 1.1x buffer, adjust the volume to 40 ml. Dissolve 10 mM β-CD by mixing or vortexing. This concentration is not very far from the solubility limit of β-CD in water, so it will dissolve slowly, and some heating (e.g. 37°C) will speed up the process.
Store frozen for the long-term. It will precipitate and require re-dissolution after thawing.

CTZ stock solution

- Relatively stable CTZ stock solutions of high concentrations can be prepared in propylene glycol (PG) and a few other organic solvents; the detailed preparation, tips and caveats are described in the Note inserts below.

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- 15 mM in propylene glycol (PG) is a convenient working stock solution which can be used to obtain a final 20 µM dilution in the water-based assay buffer (4 µl per 3 ml buffer).
- It is convenient to initially prepare a 20-80 mM stock solution in propylene glycol (depending on the initial amount and the solubility) and determine its precise concentration by absorbance (ε=10000 $L \cdot mol^{-1} \cdot cm^{-1}$) of diluted (10-30 µM) solutions in ethanol. Then this stock can be diluted to obtain the working 15 mM stock.
- The 15 mM CTZ stock should give an absorbance of 0.150 at λ=431-435 nm upon a 1:1000 dilution into absolute ethanol or methanol.
- Stocks should be stored in small aliquots at -80°C, protected from light (should be stable for many years: see the stability data in Tables 3, 6 and 9 in the following patent: Kirkland et al, 2017). **-80 °C**
- Concentrated stocks (>2-4 mM) can be considered stable when kept short-term at **Room temperature** during the preparation of the assay mix (0.5-1 h) but a long-term storage at RT (days) will lead to a slow but noticeable degradation of CTZ.
- It is crucial to precisely determine the concentration of CTZ in the stock by its absorbance using a spectrophotometer, since chemiluminescence of CTZ depends on its concentration.
- It is crucial to use the same value of extinction coefficient (ε=10000 $L \cdot mol^{-1} \cdot cm^{-1}$) to determine CTZ concentrations.

Preparation of coelenterazine stock solutions

Preparation and behavior of concentrated solutions of CTZ and its analogs in organic solvents is somewhat complicated because it depends on an interplay of solubility, solubilization and supersaturation. This is commonly encountered with many hydrophobic small molecules in drug development (Brouwers et al, 2009).

1. Propylene glycol (PG) is a recommended stock solvent for most purposes.
2. Take a tube or vial with a 0.5-2.5 mg sample of CTZ provided by the supplier (usually it is sold in small quantities).
3. Centrifuge to move most of the CTZ powder to the bottom.
4. Add the desired volume of PG and make a suspension. To obtain a 20 mM CTZ stock, use 59 µl PG per 0.5 mg CTZ and 295 µl PG per 2.5 mg CTZ.
5. In case of a glass vial it is convenient to transfer the CTZ powder into a 1.5 ml or 0.5 ml plastic microcentrifuge tube for further handling. Do this by adding a small volume of solvent, making a suspension and transferring into a new tube. This can be repeated 1-2 times until all the powder is transferred.
6. Dissolve the suspension into a (usually, supersaturated) solution according to the guidelines below.

CTZ-related compounds available from most suppliers are *crystalline* phase solids and have a poor solubility and solubilization propensity in ethanol or propylene glycol making it problematic to obtain more than 2-3 mM concentrations by prolonged mixing them at room temperature. When working with crystalline CTZ compounds, heating is required (60°C for CTZ) to dissolve them at high concentrations. This way, up to 60-100 mM (supersaturated) solutions of CTZ in EtOH or PG can be obtained. This short-term heating does not lead to CTZ degradation. These kinds of solutions are supersaturated and can precipitate to lower, thermodynamic solubility values under appropriate conditions but usually remain solubilized at room temperature if the solubilization during heating was complete.

CTZ and some of its analogs available from Nanolight (USA) are *amorphous* phase solids (described as being "freeze dried" by the manufacturer) which can usually be directly dissolved into supersaturated solutions of 60-100 mM in case of CTZ or h-CTZ at room temperature (but for bisCTZ heating is still required to obtain 6-10 mM solutions in PG and in PG/EtOH).

- Amorphous phase solids with a similar solubilization propensity can be obtained "in-house" from crystalline solids by solubilizing them first with heating in ethanol and then evaporating it quickly under vacuum (e.g. by connecting the tube top to a vacuum line).

The stock solutions should be stored at -80°C. **-80 °C**

Propylene glycol is better than ethanol in maintaining supersaturated solutions at -80°C without precipitation during long-term storage.

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Determination of the coelenterazine concentration by absorbance

CTZ is usually sold in small quantities (0.5-2.5 mg) which cannot be weighted directly and even high purity samples (>99%) can still contain a variable amount of a residual solvent. Therefore, a precise determination of CTZ concentration in a stock solution is required if results are sensitive to this factor.

Both the concentration and purity of CTZ can be easily quantified by absorbance (Fig. 5) which is an intrinsic molecular property constant in a given solvent. Absorbance can be measured in high purity ethanol or methanol. Absorbance spectra in these solvents are nearly identical. Values presented in Certificates of Analysis are usually measured in methanol. CTZ and its analogs are relatively stable in ethanol at RT (for 1-2 days) even at 10-30 µM while in methanol a noticeable degradation often occurs over 1-2 days. So, generally ethanol is more convenient to use for this purpose and it is considered to be less toxic.

1. Dilute the stock solution into ethanol to obtain an absorbance (A_{432}) of 0.05-0.2 units (5-20 µM CTZ). For 20 mM stock a 1:2000 dilution will give 10 µM CTZ.
2. Put into a spectrophotometry cuvette with a 1 cm light path.
3. Measure absorbance at 431-434 nm (the band maximum corresponding to the yellow color of CTZ solutions) using the solvent diluted into ethanol as a blank.
4. Calculate the CTZ concentration using the molar attenuation coefficient ε=10000 $L \cdot mol^{-1} \cdot cm^{-1}$.
5. Absorbance at 550 nm and 350 nm can be measured as well to assess purity.

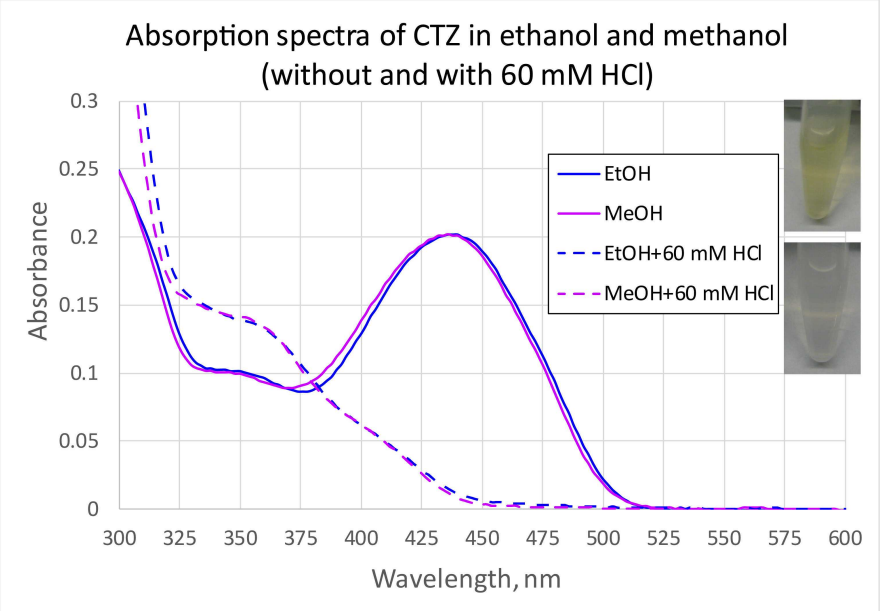

Fig. 5. Absorption spectra of coelenterazine in ethanol and methanol: either in the plain alcohols or supplemented with 60 mM HCl (acidified ethanol/methanol).

The literature ε values vary between 7600-9800 $L \cdot mol^{-1} \cdot cm^{-1}$ but for preparations of high purity it can exceed 10000 (e.g. from GoldBio: cat #CZ2.5, batch #8897.061918A; cat #C-320, batch #1789.100919A). This value is convenient to use for CTZ and most other CTZ analogs (h-CTZ, bisCTZ, furimazine, etc) with the same imidazopyrazinone core structure having the same set of conjugated double bonds which determines this yellow absorption band. A considerable variation (7600-10200 $L \cdot mol^{-1} \cdot cm^{-1}$) of ε values for CTZ and its analogs reported in the literature and in various Certificates of Analysis is likely explained by variable amounts of a residual solvent in the preparations which is not accounted for during purity determination by HPLC and other methods.

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Absorbance at 550 nm is zero for pure CTZ but increases if dehydrocoelenterazine is formed (a typical major degradation product of CTZ dissolved in various alcohols and acetone). Absorbance at 350-360 nm is lower than at 430 nm (see Fig. S3A in Eremeeva et al, 2019) but will increase if CTZ is degraded into coelenteramide: a product formed during the CTZ chemiluminescence and the major degradation product in water and in some organic solvents (e.g. DMSO, β-mercaptoethanol, etc). Formation of dehydrocoelenterazine also increases the absorbance in this range (see Fig. 5.5, 5.6 in Shimomura, 2019).

If you want to measure absorbance at the 210-280 nm range (generally not needed for purposes described here), then methanol could be preferable since it is readily available at a higher spectral purity for this spectral range (e.g. A~0.1 at 210 nm; 99.99% purity). For example,

☒ Methanol, 99.9%, for HPLC gradient grade, ACROS Organics™ **Acros Organics Catalog #AC325740010**

Organic solvents to make stock solutions of coelenterazine

This note summarizes organic solvents that can be used to make stock solutions of CTZ for different purposes.

Generally, only 3 solvents can be recommended for most purposes and the choice depends on a particular application. They are: absolute ethanol (EtOH), propylene glycol (PG) and the 50%/50% mix of EtOH/PG. Concentrated (>2-3 mM) stock solutions are stable in them when stored at -80°C (see the Tables 3, 6 and 9 in the cited patent: Kirkland et al, 2014) and can be considered operationally stable for 1-2 days even at RT.

PG is generally the best for making dilutions into water buffers and for the storage of concentrated stocks at -80C without precipitation. EtOH provides the highest solubility and stability at RT for many CTZ variants. PG/EtOH could be used for injections in mice and could be used as a solvent that balances some advantages/disadvantages of PG and EtOH.

1. EtOH provides the highest thermodynamic and supersaturation solubility of CTZ and some of its analogs. It also appears to be the most stabilizing solvent: even low (10-30 µM) concentrations are relatively stable at RT for several days, while similar CTZ solutions in methanol or isopropanol show a faster degradation at RT. The stabilization can be in part due to antioxidant impurities in the grain-derived ethanol rather than the property of the solvent itself (see below on methanol). Concentrated (supersaturated) solutions of CTZ (>20 mM) may precipitate during storage at -80°C. EtOH is volatile, so it can evaporate when handling µl volumes or at the edge of the liquid/air interface leading to a solid CTZ precipitate (film). The easy evaporation can be used for a solvent-free transfer into other organic solvents, *but such precipitates are very hard to dissolve in water buffers*, even with cyclodextrins, once the precipitate film is formed.
2. Propylene glycol is convenient since concentrated solutions generally remain soluble when stored at -80°C. Its relatively high viscosity requires slower handling but is still perfectly compatible with any micropipetting procedures. It has a lower wetting of polypropylene than EtOH or water which, together with a higher viscosity, provides an easier handling in many handling procedures involving microtubes and plastic tips (e.g. collecting by centrifugation or placing micro-liter volumes on tube walls before mixing). It is non-volatile (Võ & Morris, 2012) for practical purposes, so its micro-liter volumes won't evaporate. Low (10-30 µM) CTZ concentrations will show a noticeable degradation after 1 day at RT but millimolar stocks are relatively stable at RT for 1-2 days. Propylene glycol is compatible with osmotic pumps if needed.
3. 50% EtOH / 50% PG mix was described as one of the storage solvents by Promega (Kirkland et al, 2014) and Prolume companies (USA). This mixture provides a balance between the higher solubilization ability of EtOH, the non-precipitation of supersaturated solutions in PG and a low viscosity. The Prolume (Nanolight) company originally recommended this mixture (NanoFuel-Inject, cat # 304 NF-CTZ-FB-INJ) for intravenous and intraperitoneal mouse injections of CTZ variants as a relatively well tolerated solvent

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compared to pure ethanol or methanol. This product was later superseded by the Fuel-Inject (CAT # 303-INJ) and NanoFuel (CAT # 399) solvent products of a similar composition for *in vivo* and *in vitro* use, respectively.

Acidified methanol (or ethanol) as a historical solvent for stock solutions of coelenterazine

Historically, stock solutions of CTZ were commonly prepared in acidified methanol (Shimomura, 2019; Tannous, 2009) and later also in acidified ethanol (Charles et al, 2014). Solutions of CTZ variants in a pure methanol are often not stable during long-term storage at -20°C. Therefore, acidified methanol (with 10-60 mM HCl) was often used in which CTZ is more stable and can be stored for many months at -20°C or -80°C. Acidified methanol or ethanol is usually prepared by supplementing it with 60 mM HCl by adding 52 µl concentrated HCl (36% w/w = 12.1 M) per 10 ml of absolute alcohol. If a nearly completely water-free acidified *alcohol* solvent (i.e. methanolic or ethanolic hydrogen chloride) is desired, it can be prepared by adding 60 mM of acetyl chloride.

However, such stocks offer no apparent advantage over pure absolute ethanol, so the solvents described in the Note above should usually be used.

This lower CTZ stability in methanol compared to ethanol may be in part due to antioxidant impurities in the grain-derived ethanol. Even stability in methanol can considerably differ depending on its purity: e.g. 30 µM CTZ often remains relatively stable for several days at RT in the highly pure (ACS grade) methanol but undergoes a considerable degradation (becomes colorless) in the ultrapure (HPLC grade) methanol.

It is important to point out that in acidified alcohols CTZ is converted into another molecular form which no longer has a yellow absorption band (Fig. 5), so diluted (20-50 µM) CTZ solutions are colorless rather than yellow as usual (Fig. 5). This was noted long ago (see the Fig. 5.1 in the Chapter on coelenterazines by O. Shimomura (Shimomura, 2019)) but the identity of this form was not determined so far. The regular yellow form is generated upon dilutions of such stocks into water buffers. This conversion (color change) in the presence of HCl seem to require a solvent with a very poor hydrogen-donating properties like alcohols or acetone and it *does not* occur in water or 100% acetic acid supplemented with the same 60 mM HCl.

REFERENCES

1. Brouwers, J., Brewster, M. E., & Augustijns, P. (2009). Supersaturating drug delivery systems: the answer to solubility-limited oral bioavailability?. *Journal of pharmaceutical sciences*, *98*(8), 2549–2572. https://doi.org/10.1002/jps.21650
2. Charles, J. P., Fuchs, J., Hefter, M., Vischedyk, J. B., Kleint, M., Vogiatzi, F., Schäfer, J. A., Nist, A., Timofeev, O., Wanzel, M., & Stiewe, T. (2014). Monitoring the dynamics of clonal tumour evolution *in vivo* using secreted luciferases. *Nature communications*, *5*, 3981. https://doi.org/10.1038/ncomms4981
3. Eremeeva, E. V., Jiang, T., Malikova, N. P., Li, M., & Vysotski, E. S. (2020). Bioluminescent Properties of Semi-Synthetic Obelin and Aequorin Activated by Coelenterazine Analogues with Modifications of C-2, C-6, and C-8 Substituents. *International journal of molecular sciences*, *21*(15), 5446. https://doi.org/10.3390/ijms21155446
4. Gagnot, G., Hervin, V., Coutant, E. P., Goyard, S., Jacob, Y., Rose, T., Hibti, F. E., Quatela, A., & Janin, Y. L. (2021). Core-Modified Coelenterazine Luciferin Analogues: Synthesis and Chemiluminescence Properties. *Chemistry*, *27*(6), 2112–2123. https://doi.org/10.1002/chem.202004311
5. Kanie, S., Komatsu, M., & Mitani, Y. (2020). Luminescence of *Cypridina* Luciferin in the Presence of Human Plasma Alpha 1-Acid Glycoprotein. *International journal of molecular sciences*, *21*(20), 7516. https://doi.org/10.3390/ijms21207516
6. Kirkland, T., Wood, K. V., Hall, M., & Schwinn, M. (2017). Compositions and methods for stabilizing luminogenic substrates (*U.S. Patent No. 9,676,997*). U.S. Patent and Trademark Office. https://patents.google.com/patent/US9676997B2/e
7. Koksharov, M. (2021). Monitoring fly feeding behavior and timing by beetle luciferase reporters. *Protocols.io,* https://doi.org/10.17504/protocols.io.bs9mnh46
8. O'Kane, D. J., & Lee, J. (2000). Absolute calibration of luminometers with low-level light standards. *Methods in enzymology*, *305*, 87–96. https://doi.org/10.1016/s0076-6879(00)05479-3

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9. Loening, A. M., Wu, A. M., & Gambhir, S. S. (2007). Red-shifted *Renilla reniformis* luciferase variants for imaging in living subjects. *Nature methods*, *4*(8), 641–643. https://doi.org/10.1038/nmeth1070
10. Lucas, M., & Solano, F. (1992). Coelenterazine is a superoxide anion-sensitive chemiluminescent probe: its usefulness in the assay of respiratory burst in neutrophils. *Analytical biochemistry*, *206*(2), 273–277. https://doi.org/10.1016/0003-2697(92)90366-f
11. Luke, D. R., Tomaszewski, K., Damle, B., & Schlamm, H. T. (2010). Review of the basic and clinical pharmacology of sulfobutylether-beta-cyclodextrin (SBECD). *Journal of pharmaceutical sciences*, *99*(8), 3291–3301. https://doi.org/10.1002/jps.22109
12. Machleidt, T., Woodroofe, C. C., Schwinn, M. K., Méndez, J., Robers, M. B., Zimmerman, K., Otto, P., Daniels, D. L., Kirkland, T. A., & Wood, K. V. (2015). NanoBRET--A Novel BRET Platform for the Analysis of Protein-Protein Interactions. *ACS chemical biology*, *10*(8), 1797–1804. https://doi.org/10.1021/acschembio.5b00143
13. Maeztu, R., Tardajos, G., & González-Gaitano, G. (2010). Natural cyclodextrins as efficient boosters of the chemiluminescence of luminol and isoluminol: exploration of potential applications. *The journal of physical chemistry. B*, *114*(8), 2798–2806. https://doi.org/10.1021/jp909707x
14. Mitani, M., Sakaki, S., Koinuma, Y., Toya, Y., & Kosugi, M. (1995). Enhancement Effect of 2,6-O-Dimethyl-β-cyclodextrin on the Chemiluminescent Detection of β-D-Galactosidase Using a Cypridina Luciferin Analog. *Analytical Sciences*, *11*(6), 1013-1015.https://doi.org/10.2116/analsci.11.1013
15. Nishihara, R., Niwa, K., Tomita, T., & Kurita, R. (2020). Coelenterazine Analogue with Human Serum Albumin-Specific Bioluminescence. *Bioconjugate chemistry*, *31*(12), 2679–2684. https://doi.org/10.1021/acs.bioconjchem.0c00536
16. Shimomura, O. (2019). CHAPTER 5: The Coelenterazines. In Shimomura, O. (Eds.), *Bioluminescence. Chemical Principles and Methods* (pp. 177-198). World Scientific. https://doi.org/10.1142/9789813277113_0006
17. Schönbeck, C., Westh, P., Madsen, J. C., Larsen, K. L., Städe, L. W., & Holm, R. (2011). Methylated β-cyclodextrins: influence of degree and pattern of substitution on the thermodynamics of complexation with tauro- and glyco-conjugated bile salts. *Langmuir*, *27*(10), 5832–5841. https://doi.org/10.1021/la200381f
18. Suzuki, K., Kimura, T., Shinoda, H., Bai, G., Daniels, M. J., Arai, Y., Nakano, M., & Nagai, T. (2016). Five colour variants of bright luminescent protein for real-time multicolour bioimaging. *Nature communications*, *7*, 13718. https://doi.org/10.1038/ncomms13718
19. Szente, C., & Fenyvesi, E. (2017). Cyclodextrin-Lipid Complexes: Cavity Size Matters. *Structural Chemistry*, *28*(2), 79–492. https://doi.org/10.1007/s11224-016-0884-9
20. Szente, L., Singhal, A., Domokos, A., & Song, B. (2018). Cyclodextrins: Assessing the Impact of Cavity Size, Occupancy, and Substitutions on Cytotoxicity and Cholesterol Homeostasis. *Molecules*, *23*(5), 1228. https://doi.org/10.3390/molecules23051228
21. Tannous B. A. (2009). *Gaussia* luciferase reporter assay for monitoring biological processes in culture and *in vivo*. *Nature protocols*, *4*(4), 582–591. https://doi.org/10.1038/nprot.2009.28
22. Teranishi K. (2007). Luminescence of imidazo[1,2-a]pyrazin-3(7H)-one compounds. *Bioorganic chemistry*, *35*(1), 82–111. https://doi.org/10.1016/j.bioorg.2006.08.003
23. Teranishi, K., & Shimomura, O. (1997a). Coelenterazine analogs as chemiluminescent probe for superoxide anion. *Analytical biochemistry*, *249*(1), 37–43. https://doi.org/10.1006/abio.1997.2150
24. Teranishi, K, & Shimomura O. (1997b). Solubilizing Coelenterazine in Water with Hydroxypropyl-β-cyclodextrin. *Bioscience, Biotechnology, and Biochemistry*, *61*(7), 1219-1220. http://dx.doi.org/10.1271/bbb.61.1219
25. Varga, E., Benkovics, G., Darcsi, A., Várnai, B., Sohajda, T., Malanga, M., & Béni, S. (2019). Comparative analysis of the full set of methylated β-cyclodextrins as chiral selectors in capillary electrophoresis. *Electrophoresis*, *40*(21), 2789–2798. https://doi.org/10.1002/elps.201900134
26. Vassel, N., Cox, C. D., Naseem, R., Morse, V., Evans, R. T., Power, R. L., Brancale, A., Wann, K. T., & Campbell, A. K. (2012). Enzymatic activity of albumin shown by coelenterazine chemiluminescence. *Luminescence*, *27*(3), 234–241. https://doi.org/10.1002/bio.2357
27. Viviani, V. R., & Ohmiya, Y. (2006). Bovine serum albumin displays luciferase-like activity in presence of luciferyl adenylate: insights on the origin of protoluciferase activity and bioluminescence colours. *Luminescence*, *21*(4), 262–267. https://doi.org/10.1002/bio.916
28. Wang, K. Y., Wu, C., Shimajiri, S., Enomoto, T., Kubota, H., Akiyama, H., & Ohmiya, Y. (2020). Quantitative immunohistochemistry using an antibody-fused bioluminescent protein. *BioTechniques*, *69*(4), 302–306. https://doi.org/10.2144/btn-2020-0006
29. Yoshita, M., Kubota, H., Shimogawara, M., Mori, K., Ohmiya, Y., & Akiyama, H. (2017). Light-emitting-diode Lambertian light sources as low-radiant-flux standards applicable to quantitative luminescence-intensity imaging. *The Review of scientific instruments*, *88*(9), 093704. https://doi.org/10.1063/1.5001733

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BEFORE STARTING

Prepare the required materials.
Read the guidelines explaining the basis and the scientific background of this reference method.

1 Prepare for the experiment.

1.1 Bring the reference assay solutions (with 10 mM β-CD and/or with 10 mM TMCD) to room temperature.

1.2 Turn on the luminescence plate reader or luminometer, warm it up and make it ready for the measurements.

1.3 Take out a small (20-40 µl) aliquot of 15 mM CTZ in propylene glycol from **-80 °C** and allow it to reach **Room temperature** in the dark.

The 15 mM CTZ stock of the correct concentration should give an absorbance of 0.150 at λ=431-435 nm upon a 1:1000 dilution into absolute ethanol or methanol (ε=10000 $L \cdot mol^{-1} \cdot cm^{-1}$).

Preparation of the luminescence reference mixture

2 Prepare a tube to make a desired volume of the reference luminescence solution.

1.5-2 ml tubes can be used for smaller volumes and 12 ml tubes can be used for larger volumes.

For example, add 1.5 ml 10 mM cyclodextrin assay solution to a 1.5-2 ml tube or add 3 ml to a 12 ml tube.

3 Calculate the amount of the 15 mM CTZ stock required to obtain the 20 µM final solution in the assay mix.

For example, use 2 µl of stock solution for the 1.5 ml volume and 4 µl for the 3 ml volume.

The presence of 18 mM propylene glycol (obtained as a result of the 1:750 dilution of the CTZ stock made in propylene glycol into a water-based assay buffer) does not affect the chemiluminescence values when compared with a vehicle-free 20 µM CTZ solution. The latter can be obtained by evaporating the required µl volume of a CTZ stock made in ethanol and its re-dissolution in the cyclodextrin assay buffer by a thorough mechanical mixing.

4 Place the required small volume of the 15 mM CTZ stock above the level of the buffer as several micro-drops (on the tube walls or under the cap).

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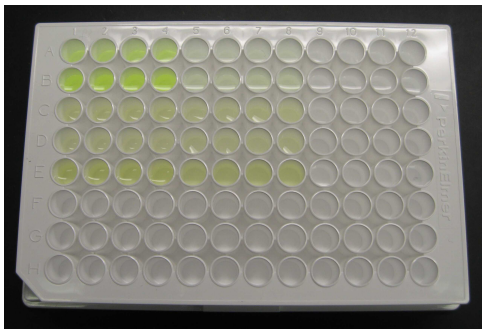
0.93 µl volumes of 15 mM CTZ stock (in propylene glycol) are placed under caps of 1.5 ml microtubes containing 700 µl buffer - for subsequent rapid mixing by vortexing.

5 Close the tube and mix by vigorous vortexing and shaking.
Make sure that CTZ is fully solubilized.

- In the presence of 10 mM β-CD or TM-β-CD the 20 µM concentration of CTZ is nominally soluble and should go into solution after a rapid vortex mixing. If using a solid CTZ residue, e.g. after evaporation (rather than micro-droplets in PG or other solvent), then a thorough mechanical mixing may be required to bring it into solution even in the presence of cyclodextrins.
- In the absence of cyclodextrins or other solubilizers (e.g. serum) in water buffers, 10-20 µM CTZ is in the supersaturated concentration range at pH 7.40 (above the thermodynamic solubility), so some yellow crystals can occasionally be observed if mixing was not rapid enough.

6 Quickly dispense the final assay mix into the microplate wells or tubes which will be used for luminescence measurements.



An opaque white 96-well plate with samples.
rows C,D: 100 µl of 20 µM CTZ in 50 mM Na-phosphate buffer (pH 7.40) containing 10 mM TMCD (columns 1-4) or 10 mM β-CD (columns 5-8);
row E: the same as in rows C,D but 200 µl;
rows A-B: 5 and 0.5 µM fluorescein in 0.1 M NaOH (used as a fluorescence reference in a separate experiment);

Luminescence measurements

7 Start to measure luminescence within the 5-10 min after mixing CTZ with the assay solution, because chemiluminescence will slowly decay.

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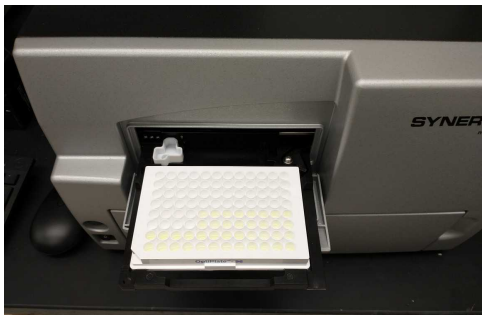
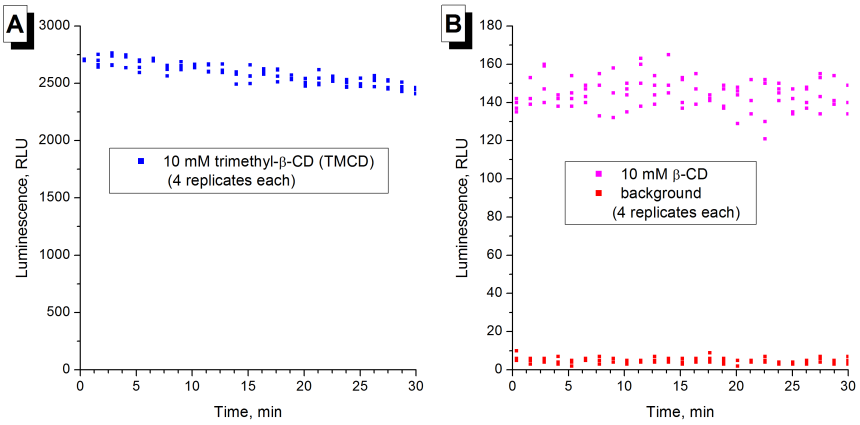


96-well plate in the loading tray of the plate reader.

A single-tube luminometer.

Chemiluminescence signal can be averaged for the first 10-20 min for TMCD and 30 min for β-CD.

The chemiluminescence signal slowly decays (as CTZ is slowly oxidized) but can be considered stable for practical purposes during the first 10-20 min with 10 mM TMCD and 30-50 min for 10 mM β-CD (see the Figure below).



Time profiles of luminescence decay during the first 30 min for 20 µM CTZ dissolved in the presence of 10 mM TMCD (A) or 10 mM β-CD (B) in 50 mM Na-phosphate buffer (pH 7.40). The recording was performed on Synergy HTX platereader (Biotek, USA) in a 96-well white opaque plate (Perkin Elmer, USA). The PMT detector gain was 135 (a default value). See also the Table 1.

- Chemiluminescence of CTZ with TMCD is about 20 times higher than with β-CD making TMCD a more convenient luminescence standard.
- The two reference solutions can be used to complement each other as two independent reference standards with the different light output.
- With not very sensitive instruments, the chemiluminescence with β-CD can be not very far from the detection limits making in some cases its use problematic.
- β-CD is available at low prices and from a wider number of suppliers compared to TMCD
- A cheaper RMCD (randomly methylated β-CD) can be used in place of TMCD for routine reference measurements, since it leads to a similar light output. However, it must be initially referenced against TMCD/β-CD because this randomly methylated compound can differ considerably lot to lot.

How the signal varies depending on differences (i.e. uncertainties and errors) in the composition of the assay mix:

- the signal depends on CTZ concentration with a slightly less than proportional or semi-saturating response which depends on the type of cyclodextrin (see the Guidelines section)

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- the response is less than proportional relative to the cyclodextrin concentration (see the Guidelines section)
- changing pH by -0.20 and +0.20 units from pH 7.40 results in approximately 17% decrease or 20% increase in chemiluminescence, respectively.

8 Use the measured luminescence values (usually, the relative light units per second, RLU/sec) as a reference standard to calibrate the RLU values of the instrument relative to other instruments or to variations in the values generated on the same instrument at different dates due to changes in its operation.

If the same reference standard is measured on another instrument, then the RLU values produced by these instruments can be directly compared by calculating an appropriate conversion coefficient.

The Table 1 below shows the reference values obtained for the Biotek HTX platereader in this work.

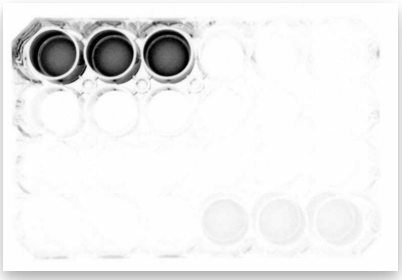
Examples of reference values that can be obtained.

A	B	C	D	E	F	G
Plate	Volume per well	Probe	10 mM CD	Averaging time, min	Luminescence, RLU/sec (corrected for the background)	Background, RLU/sec (uncorrected)
					PMT gain = 135	
24-well CytoOne (transparent)	0.5	Bottom	β-CD	30	38 ± 4	14 ± 3 (*a) 4.4 ± 1.7 (*b)
			TMCD	10	932 ± 56	
96-well Nunc (black walls, clear thin bottom) (with lid on top)	0.1	Bottom	β-CD	20	70 ± 7	8 ± 2
			TMCD	10	1325 ± 27	
		Top	β-CD	20	18.1 ± 3.3	4.8 ± 1.3
			TMCD	10	319 ± 14	
96-well CulturePlate, OptiPlate (white non-transparent) (no lid on top)	0.1	Top	β-CD	30	140 ± 8	4.1 ± 1.4
			TMCD	10	2670 ± 45	
	0.2		β-CD	30	231 ± 11	
			TMCD	10	4338 ± 59	

Table 1. Reference RLU values of the CTZ/cyclodextrin standards obtained on the Biotek HTX plate reader with different plates. The PMT gain was set to 135 (default value) in all measurements. The instrument can measure luminescence either with the bottom or the top fiberoptic probe collecting the emitted light. The probe was located at the well center (below or above). The top probe height was 3.1 mm above the top surface (of the plate plate or lid if the latter was used).
*a - the background signal of the plate with an empty moat; *b - the background signal of the plate with a moat filled with 30 ml black agar gel (2% charcoal powder, 1% agar) to reduce the bioluminescence cross-talk between transparent wells;

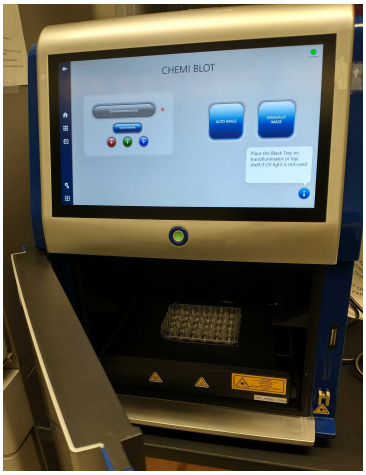
The chemiluminescence of these reference solutions can also be used to assess and reference the sensitivity of imaging instruments. Below is an example of a weak chemiluminescence of these solutions captured on Azure 600 imager (Azure Biosystems, USA). A transparent CytoOne 24-well plate contained 500 µl samples in the following wells: TMCD reference solution in A1-A3, β-CD reference solution in D4-D6, a plain buffer in C1-C3 and all other wells were left empty. The plate was imaged for 300 sec using the chemiluminescence mode with the 3x3 pixel binning. The instrument obtains 32-bit tiff images which have an intensity range of white from 0 (black) to 65535. The image in the panel "A" was saved as jpeg file without any adjustments, while in the panel "B" the intensity was trimmed to the 63742-65535 range to highlight the observed chemiluminescence.

Citation: Misha Koksharov (07/12/2021). Chemiluminescence of coelenterazine catalyzed by cyclodextrins as a luminescence reference standard for luminometers . https://dx.doi.org/10.17504/protocols.io.bwh2pb8e



A) Unadjusted 32-bit image (300 sec exposure, 3x3 pixel binning).
Range of white: 0-65535.

B) The same image adjusted.
Range of white: 63742-65535.



Azure 600 imager with a 24-well plate inside.

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