Title: The mRNACalc webserver accounts for the hypochromicity of modified nucleosides and enables the accurate quantification of nucleoside-modified mRNA.

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Abstract:

Nucleoside-modified mRNA technologies necessarily incorporate N1-methylpseudouridine into the mRNA molecules to prevent the over-stimulation of cytoplasmic RNA sensors. Despite this modification, mRNA concentrations remain mostly determined through the measurement of UV absorbance at 260 nm wavelength (A_{260}). Herein, we report that the N1-methylpseudouridine absorbs approximately 40% less UV light at 260 nm than uridine, and its incorporation into mRNAs leads to the under-estimation of nucleoside-modified mRNA concentrations, with 5-15% error, in an mRNA sequence dependent manner. We therefore examined the RNA quantification methods and developed the mRNACalc webserver. It accounts for the molar absorption coefficient of modified nucleotides at 260 nm wavelength, the RNA composition of the mRNA, and the A_{260} of the mRNA sample to enable accurate quantification of nucleoside-modified mRNAs. The webserver is freely available at https:\\www.mrnacalc.com.

Introduction:

The therapeutic use of messenger RNA (mRNA) has sparked great optimism in the development of novel vaccines and therapeutics against a myriad of infectious or yet incurable diseases.[1] The mRNA technology enables the production of antigenic, functional, and/or therapeutic proteins by introducing mRNA into the human body and cells.[2] Since mRNAs act in the cytoplasm transiently, they do not bear any risk of integration into the host cell genome. Most importantly, the mRNA technology enables rapid, cost-efficient, and scalable production, which is free of cellular (cell cultures) or animal materials.[3] Thus, mRNA technologies facilitate manufacturing and allow for a rapid response to emerging infectious diseases, as emphatically underscored by the rapid rollout of COVID-19 mRNA vaccines in many parts of the world. Modified nucleosides, such as pseudouridine (Ψ), N1-methylpseudouridine ($m^1\Psi$), and 5-methylcytidine (m^5C), are often incorporated into the mRNA molecules. Such modifications reduce stimulation of cytoplasmic RNA sensors, such as toll-like receptors 3 and 7, for improved safety profiles and enhanced mRNA translation.[4,5] However, how modified nucleosides affect mRNA concentration measurements and potentially confound pre-clinical dosing, efficacy, and toxicology studies, which could make or break further clinical development of any therapeutic, remains undefined.

The determination of RNA concentration often relies on measurements of its UV absorbance at 260 nm wavelength (A_{260}) and the implementation of the Beer–Lambert law.[6] The accuracy of these measurements is scattered by the variable hypochromicity of RNA due to its sequence-dependent folding. The molar absorption coefficient (MAC or extinction coefficient, ε) of a folded RNA at 260 nm (ε_{260}) is reduced as compared to its unfolded state.[7] This difference is buffer- and concentration-dependent and arises from changes in the chemical environment of the nucleobases – the main

chromophore, due to base-pairing, stacking, intermolecular interactions, and other conformational changes. Considering these variabilities, a rough estimation for the MAC_{260} of any single stranded RNA (ssRNA), 40 µg/ml per absorbance unit, is extensively used and its associated ±10–20 % error in the estimation of RNA concentration is widely accepted.[6] This error range may suffice to assess dose-response for mRNA therapeutics across several orders of magnitude *in cellula* or *in vivo* experiments. Yet it would be valuable to know concentrations at higher accuracy for the development of mRNA technologies. Our particular concern is in measurements of self-amplifying RNAs (saRNA) and nucleoside-modified mRNAs. The logarithmic amplification of saRNA can convert a 20 % accepted error in RNA concentration into several-fold differences in dose-response between one experiment and the subsequent replicates. The chemical modifications on the nucleobases of mRNA can also induce profound changes in the mRNA MAC, hindering the accurate quantification of nucleoside-modified mRNA concentrations.

To attain greater accuracy in RNA quantification, RNA molecules are hydrolysed prior to UV absorbance determination using a combination of thermal and alkaline hydrolysis. [6,8] The RNA hydrolysis shifts the hypochromic folded state of the RNA to the hyperchromic state of the single monophosphate nucleotides.[9] Since the precise MAC of the four standard nucleotides in aqueous buffered solution is known, the molar absorption of any hydrolysed mRNA can be calculated as the sum of the molar absorption of its nucleotide compositions. Thus, upon the A_{260} determination, the RNA concentration can be quantified with an error of ~ 4 % using these methods.[6] The incorporation of modified nucleosides can alter the RNA molar absorption and increase the error of the measurements in an RNA sequence-dependent manner. Other non-UV-spectroscopic methods relying on the unspecific RNA binding of

77 fluorophores for the determination of RNA concentration may help to overcome any
78 change in the MAC of modified-nucleoside mRNA. However, the impact of RNA
79 modifications on the binding affinity of these fluorophores also remains unknown.

80 Herein, we report our effort to revisit and determine the MAC of modified nucleosides
81 (Ψ, $m^1\Psi$, and m^5C). We also examined three different methods for RNA hydrolysis and
82 provided them along with the mRNACalc webserver. This web tool incorporates the
83 most recently revised MAC_{260} for standard, modified, and mRNA capping nucleosides,
84 allowing the accurate determination of standard and nucleoside-modified mRNAs
85 using UV spectroscopy.

Results:

To assess the impact of chemical modifications on the spectrophotometric parameters of pyrimidine nucleosides for mRNA quantification, we determined and compared the molar UV absorption curves of standard nucleosides (U and C) and the modified nucleosides that have recently been employed in nucleoside-modified mRNA technologies (Ψ, $m^1Ψ$, and m^5C). For the cytidine to 5-methylcytidine comparisons, a shift of +7 nm in the peak maximum ($\Delta\lambda_{max}$) was observed with a 20.8 % reduction in the ε_{260} for the m^5C nucleoside (Figure 1A and 1B). For the Ψ and $m^1Ψ$ curves, a similar shift was detected ($\Delta\lambda_{max}$ = +9 nm in $m^1Ψ$, Figure 1C), with a reduced molar absorption at 260 nm for $m^1Ψ$ ($\Delta\varepsilon_{260}$ = -22.8 %). More importantly, $m^1Ψ$ is hypochromic as compared to uridine at λ_{max} ($\Delta\varepsilon_{max}$ = -21 %) and, due to the λ_{max} shift, $m^1Ψ$ absorbs 39.8 % less than uridine at 260 nm (Figure 1D), suggesting that $m^1Ψ$-incorporated mRNAs can have reduced molar absorption coefficients.

To assess whether the complete U-to-$m^1Ψ$ substitution alter the UV absorbance of an mRNA, the same mRNA was transcribed using either U, Ψ, or $m^1Ψ$. These mRNA also encoded a dimeric-Broccoli (dBroc) aptamer in their 3' untranslated region (Figure 2A). Once the DFHBI-1T fluorophore was bound to the G-quadruplex in the Broccoli aptamer, the mRNA emitted green light upon excitation.[15] We also confirmed that the brightness, melting point, and affinity of the DFHBI-1T-Broccoli complex are not significantly perturbed by the U-to-Ψ or U-to-$m^1Ψ$ substitutions (Table S1 and Figure S1). After normalizing the UV absorbance (A_{260}) of each mRNA by its corresponding fluorescence (F_{507}), it was observed that in practice the relative UV absorbance of the nucleoside-modified mRNA was significantly reduced as compared to the standard mRNA (ΔA_{260} = -10.6 %, Figure 2B and 2C). This hypochromicity was also

110 independently observed in two additional mRNAs with either higher or lower $m^1\Psi$
111 composition (ΔA_{260} = -11.8%, and -6.7%, respectively in Figure 2C). These findings
112 confirmed that $m^1\Psi$-mRNAs are hypochromic and their hypochromicity is dependent
113 on the nucleoside composition. To correct for the observed hypochromicity in
114 nucleoside-modified mRNA, we built the mRNACalc software that calculates the
115 expected MAC_{260} of a hydrolysed mRNA. It considers its nucleotide composition and
116 the MAC of standard and modified nucleosides, including the nucleosides in the mRNA
117 cap (Documentation in Text S1 and Tables S2-S6). We used this software to predict
118 MAC_{260} for the different U-, Ψ-, and $m^1\Psi$-dBroc-mRNAs in Figure 2C and plotted their
119 Ψ-/U-mRNA and $m^1\Psi$-/U-mRNA MAC_{260} ratio against the experimentally determined
120 normalized A_{260}/F_{507} ratio (Figure 2D). The observed linearity in this graph
121 corresponds to the expected linearity in the Beer-Lambert law for standard and
122 modified nucleosides and its implementation in ssRNAs, such as mRNA.

123 To enable accurate measurement of nucleoside-modified mRNA, we also assessed
124 different RNA hydrolysis methods. The modern analytical use of alkaline hydrolysis of
125 RNA is known since 1922, when Steudel and Peiser demonstrated that 1 M NaOH
126 hydrolysed yeast RNA, whereas thymus DNA resisted the NaOH hydrolysis.[17] The
127 alkali-promoted transesterification of RNA occurs due to the nucleophilic attack of the
128 2'-OH in the ribose to the 3',5'-phosphodiester bond, explaining the alkali-resistance
129 of the 2'-deoxyribonucleotides (Figure 3A).[18] This reaction is further catalysed with the
130 introduction of heat. However, the combination of thermal and alkaline hydrolysis, e.g.,
131 1 M NaOH at 95 °C, also catalyses the deamination of cytosine to uridine in a small
132 percentage of residues.[19,20] Thus, a compromise between the two methods is often
133 applied. In our hands, three of such protocols showed a similar increase in A_{260} upon
134 hydrolysis of yeast RNA – a historical standard sample for these methods (Figure 3B).

One of these methods (0.8 M NaOH at 37 °C) was also applied on U- and $m^1\Psi$-mRNAs (Figure 3C), the use of RNA hydrolysis indeed increased the A_{260} of both types of mRNA, confirming the importance of performing RNA hydrolysis to remove the effect of RNA folding on the mRNA UV absorption and therefore allow a more accurate determination of mRNA concentrations. We also applied the RNA hydrolysis methods on U- and $m^1\Psi$-mRNAs and determined their concentration by measuring their A260 and using the mRNACalc software to correct for hypochromicity. The concentration of these mRNAs was then reassessed by performing direct A_{260} measurements, without prior RNA hydrolysis and implementing the extensively used MAC_{260} for ssRNA (40 µg/ml per absorbance unit), or by using a commercially available fluorescence-based assay. We could observe that both methods differentially estimated the nucleoside-modified and standard mRNA concentrations, with an underestimation of the $m^1\Psi$-mRNA concentration (Figure S2).

Discussion:

Pseudouridine is an isomer of uridine – the standard nucleoside in RNA. Pseudouridine, as opposed to other nucleosides, is a carbon-carbon ribofuranosyl nucleoside, i.e., the uracil nucleobase is linked to the ribose through its fifth carbon, instead of an N1-linkage.[10] This unique arrangement places the N1-imino group toward the so-called “C-H” edge of the pyrimidine ring and confers additional properties to this edge in pseudouridine. This imino hydrogen proton is susceptible to hydrogen bonding, chemical exchange, and chemical modifications such as N1-methylation. Thus, the N1-methylpseudouridine, as well as the m^5C, represents a modification of the C-H edge of the pyrimidine nucleobase. The influence of a 5-methyl substituent on the UV molar absorption of pyrimidine rings is well known since the 1940’s when Sister Miriam Michael Stimson showed that a similar 5-methyl

modification also differentiates uridine from thymidine and provokes a subtle reduction in molar absorbance (ΔMAC_{max} = -3 %) and a shift of the peak maximum ($\Delta\lambda_{max}$ = +5 nm) to a longer wavelength – a bathochromic shift.[11–14] In combination, these two effects provoke a substantial MAC_{260} reduction for the thymidine nucleoside (ΔMAC_{260} = -11.4 %). In our study, similar differences were observed for the C-to-m^5C and Ψ-to-$m^1\Psi$ comparisons, with a more pronounced MAC_{260} difference for the U-to-$m^1\Psi$ comparison. Thus, the substitution of uridine by $m^1\Psi$ in mRNA technologies can substantially modify the spectrophotometric properties of the mRNA.

In principle, the modified nucleosides may also promote mRNA folding and reduce its UV absorption. This is particularly relevant for the pseudouridine modification. Its N1-hydrogen can engage in additional hydrogen bonds, promoting and stabilizing RNA folding. For instance, the U-to-Ψ substitution in tRNA stabilizes the folded structure that is essential for translation.[16] However, the $m^1\Psi$ nucleobase lacks this additional hydrogen bonding capability, and it is expected to have little or no effect on the RNA folding of less structured RNA molecules such as mRNAs. Considering that both Ψ- and $m^1\Psi$-mRNAs followed the anticipated hypochromicity that is associated with the modified nucleosides' hypochromicity at 260 nm wavelength (Figure 1) and their abundance in the mRNA (Figure 2C), rather than the expected distinct contribution of Ψ and $m^1\Psi$ to RNA folding, we can conclude that the observed reduction in the UV absorption of nucleoside-modified mRNA is mainly determined by the nucleobase composition and the intrinsic MAC of the nucleosides in the purified mRNAs. Importantly, the UV absorption spectrum of the $m^1\Psi$-mRNA also depicted a broad absorption peak and a bathochromic shift, which brings additional implications for the assessment of the RNA sample purity (Figure 2B and Text S2). These findings indicate

that, for accurate determination of nucleoside-modified mRNA concentrations and proper interpretation of dose-ranging preclinical studies, the reported UV spectroscopic differences must be accounted for. Otherwise, nucleoside-modified mRNA concentrations may be underestimated by 5 to 15 %, depending on the proportion of $m^1\Psi$ in the mRNA composition.

Considering that traditional methods underestimate the nucleoside-modified mRNA concentrations and to ease the implementation of the reported UV absorption parameters, we provide the mRNACalc software as an open-source webserver to calculate the MAC_{260} for nucleoside-modified mRNAs. It accounts for the hypochromicity of modified nucleosides as well as for the nucleoside composition of the mRNA, including the mRNA cap. Once the RNA sequence, the A_{260}, and the RNA stock volume values are provided as input, the mRNACalc webserver calculates the RNA stock concentration in nM and ng/µl and the total RNA mass in µmole and µg. The webserver also includes the revisited experimental protocols and a workflow that implements a linear regression model from multiple measurements at serial dilutions (Figure 4). This workflow aims at reducing the impact of sample handling variation. Hence, the mRNACalc webserver represents a freely available and all-inclusive tool for the determination of nucleoside-modified mRNA concentrations using UV spectroscopy.

Materials and Methods:

The Beer-Lambert experiments

Pseudouridine (≥ 98% purity), 5-methylcytidine (≥ 99% purity), Cytidine (99% purity) and Uridine (99% purity) were purchased from Sigma-Aldrich. N1-methylpseudouridine (>95% purity) was purchased from Biosynth Carbosynth. They were used as received. Phosphate buffer solutions with a total phosphate concentration of 16 mM from monosodium and disodium phosphate salts dissociated in ultrapure water (Milli-pore) were freshly prepared on the day of each experiment. The pH of the solution was adjusted using 0.1 M solutions of NaOH and HCl to the desired pH of 7.4 (± 0.1 pH units). Steady-state absorption was recorded using a Cary 100 spectrometer. Serial dilutions of known concentration were carried out such that the absorbance reading at the respective lambda maximum (local maximum absorbance) remained below 1.0, within the linear range of the instrument. The MACs were experimentally determined using the slope from the linear regression from plotting absorbance versus concentration. The correlation constant for the linear regression analysis of the Beer-Lambert's Law data for determining molar absorption constants was >0.9999 showing a strong linear relationship.

mRNA *in vitro* transcription and purification

The Plasmid DNA template (pUCIDT plasmid) was grown in DH5 alpha *E. coli* (New England Biolabs, Inc.) in 300 ml of Luria-Bertani broth supplemented with Kanamycin (50 μg/ml) and a maxi preparation was performed using the QIAGEN® Plasmid Plus Maxi Kit following manufacturer instructions. The plasmid-encoded a T7 promoter followed by the mCherry gene with a degradation tag (1449 nucleotides) plus the 3' and 5' untranslated regions (UTR) of the BNT162b2 mRNA vaccine (541 nucleotides).

The double broccoli aptamer was encoded within the poli-adenine region in the 3'UTR. The plasmid was linearized by EcoRV restriction enzyme digestion at the end of the 3' UTR.

A standard T7 transcription reaction included 30 mM Tris-HCl, pH 7.9, 2 mM spermidine, 30 mM $MgCl_2$, 5 mM NaCl, 10 mM DTT, 50 µg/ml BSA (New England Biolabs, Inc.), 0.005% Triton X-100, 2% polyethylene glycol (PEG8000), 5 mM of each triphosphate ribonucleotide (standard nucleotides were purchased from Jena Bioscience GmBH and pseudouridine and N1-methylpseudouridine from BOC sciences), 2 µM linearized plasmid DNA template, 3.5 µM T7 RNA Polymerase (in house produced and purified) and 0.0025 units of *E. coli* inorganic PPase (New England Biolabs, Inc). All reagents were purchased from Sigma-Aldrich, unless otherwise stated. The reactions were incubated at 37 °C for 2.5 hours and stopped by the addition of 500 mM EDTA, pH 8 to a final concentration of 35 mM.

The mRNA was purified using anion exchange chromatography. A PRP-X600 Anion exchange column (Hamilton Company, Inc.) was equilibrated in Buffer A (85:15 100 mM TRIS, pH 8/Acetonitrile). RNA samples were loaded onto the column at a flow rate of 3 ml/min and eluted with a 40-minute gradient of 0-40% buffer B (85:15 100 mM TRIS 2.5 M LiCl, pH 8/Acetonitrile). Fractions containing the mRNA were collected and the mRNA molecules were precipitated using standard Butanol extraction.[21] The purity of the mRNA preparation was assessed using high-resolution automated electrophoresis in the Agilent 2100 Bioanalyzer system using the Bioanalyzer RNA 6000 pico assay (Agilent Technologies, Inc).

Determination of the mRNA UV absorption spectrum

To determine the UV absorption spectrum of mRNAs, the mRNAs stocks were diluted to approximately 25 nM into a buffer containing 40 mM HEPES pH 7.4, 5 mM $MgCl_2$, and 100 mM KCl to a final volume of 2 ml. Five independent mRNA samples were prepared per mRNA set (U-, Ψ-, and $m^1Ψ$-mRNA). The UV absorption spectra were recorded for each mRNA sample using in a UV-3600i plus UV-VIS spectrophotometer (Shimadzu Corp.).

Excitation-emission experiments on the DFHBI-1T bound mRNAs

After UV absorption determination, the mRNA samples were bound to the DFHBI-1T fluorophore, by adding 100 μM DFHBI-1T, 100% DMSO to a 500 nM concentration into the 2-ml mRNA samples. Fluorescence was measured using a Fluorolog-3 spectrofluorometer (Horiba Scientific) using the excitation and emission wavelengths commonly used for DFHBI-1T (Excitation: 472 nm, emission: 507 nm).[15]

Determination of the relative UV absorbance (A_{260})

The A_{260}/F_{507} ratios were calculated for each mRNA sample. The mean A_{260}/F_{507} values for U-, Ψ-, and $m^1Ψ$-mRNA were calculated. The A_{260}/F_{507} values of each sample were normalized using the mean A_{260}/F_{507} value from the U-mRNA as a reference and they were plotted in a dot plot. The t-tests were applied to compare the mean A_{260}/F_{507} values across each pair of mRNA sets, using a p-value of 0.005 as a cut-off of significance.

Methods of RNA hydrolysis

Two methods of RNA hydrolysis were tested in this study. Torula yeast RNA was used as a standard RNA sample (Sigma-Aldrich). The Yeast RNA stock was prepared at 1000 μg/μl in water. Thus, after 1/25 dilution, the UV absorbance of this RNA sample

would be within the linear range of the instrument (UV-3600i plus UV-VIS spectrophotometer, Shimadzu Corp.).

The most extensively used alkaline RNA hydrolysis method involves adding 1 part of RNA and 4 parts of 1 M NaOH and incubating them at 37 °C for 1 hour.[22] To test this method, twelve yeast RNA samples were hydrolysed. Every 10 minutes, a sample was neutralised with 4 parts of 1 M HCl and diluted to 1/25 with 16 parts of water. Three UV absorbance measurements were performed on every sample. Similarly, a room temperature variation of this method is often used for overnight RNA hydrolysis. Therefore, twelve RNA samples were hydrolysed and incubated at 20 °C for up to 15 hours. Samples were neutralized and diluted hourly followed by three UV absorbance measurements.

A second method of thermal hydrolysis at neutral pH was also tested.[8] To test this method, twelve yeast RNA samples hydrolysed (1 part of RNA in 9 parts of 60 mM Na_2CO_3 pH 8) with an incubation at 95 °C for up to 2 hours. Every 20 minutes, a sample was diluted to 1/25 with 15 parts of water, and three UV absorption measurements were performed on every sample.

Data and code availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. The webserver is available at https:\\www.mrnacalc.com. The website is free and open to all users and there is no login requirement. The html script for the mRNACalc webserver is available under GNU general public licence from https://github.com/estebanfbfc/mRNACalc. It can be downloaded free of charge and run locally without internet access.

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Author Contributions

E.F. conceived the study. E.F. and C.E.C-H. supervised the project. E.F. developed the mRNACalc webserver. S.E.K. and S.J.H. performed the Beer-Lambert experiments and prepared the corresponding figure panel. E.F. and X.L. performed the relative absorbance of mRNA experiments and analysed the data. E.F. prepared figures, wrote the initial draft of the manuscript and edited the submitted version of the manuscript with contributions from all the authors.

Declaration of Interests

Authors declare no competing interests.

Keywords

N1-methylpseudouridine, pseudouridine, modified-nucleoside, mRNA, UV absorption.

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List of Figure Captions

Figure 1: The nucleobase methylation and its bathochromic effect on the UV molar absorption spectra of pyrimidines.
(A) Skeletal formula of uridine, thymidine, cytidine, 5-methylcytidine, pseudouridine and N1-methylpseudouridine. The methyl substituents are highlighted in red. These λ_{max}, ε_{max} and ε_{260} values are implemented in the mRNACalc webserver. The source of these values is provided in the supplemental material. (B) Steady-state absorption spectra of cytidine (black line) and 5-methylcytidine (red line) at pH 7.4. (C) Steady-state absorption spectra of pseudouridine (orange line) and N1-methylpseudouridine (green line) at pH 7.4. (D) Steady-state absorption spectra of uridine (light blue line) and N1-methylpseudouridine (green line) at pH 7.4. The ε_{260} for U and $m^1\Psi$ are shown.

Figure 2: The hypochromicity of nucleoside-modified mRNA can be predicted from their nucleoside composition.
(A) Schematic representation of the mRNAs that were designed to determine the normalized A_{260}/F_{507} values (B) Relative UV absorption curves from mRNAs with uridine or N1-methylpseudouridine nucleosides. They were normalized to the corresponding F507 values and plotted relative to the peak maximum of the U-mRNA. (C) The normalized A_{260}/F_{507} values from five replicates of the U-, Ψ-, and $m^1\Psi$-mRNAs are shown for dBroc-mRNA1. Similar measurements in two additional U-, and $m^1\Psi$-mRNAs are shown. the black lines correspond to the average absorbance. Values are relative to the average absorbance of the U-mRNA. All comparisons of the mean relative A_{260}/F_{507} values were significant (t-test; $p< 0.005$). (G) The normalized A_{260}/F_{507} values in Figure 2C were plotted against their predicted hypochromicity using the mRNACalc software.

Figure 3: RNA hydrolysis is essential for the determination of mRNA concentrations.
(A) Alkali-promoted transesterification allows RNA hydrolysis and mRNA quantification. Under alkaline conditions, the reactive -OH triggers the nucleophilic attack of the 2'-OH on the 3',5'-phosphodiester linkage, converting the ground-state configuration of RNA into a penta-coordinated intermediate and leading to a 2'3'-cyclic phosphodiester. This cyclic form is then known to form 3' and 2' monophosphate nucleotides (not shown). (B) Thermal and/or alkaline hydrolysis of RNA over time. Yeast RNA was hydrolysed using three different previously described methods and the ΔA_{260} was determined using an UV spectrophotometer at different intervals. For expedite RNA hydrolysis (1- or 2-hours incubation), a combination of thermal and alkaline hydrolysis can be used (dark blue dots, 0.8 M NaOH at 37 °C; red dots, 0.5 M Na_2CO_3 pH 8 at 95 °C). For overnight incubation, alkaline hydrolysis suffices (light blue dots, 0.8 M NaOH at 20 °C, the last four measurements were performed after an overnight incubation). Dots indicate the mean value of three measurements. Error bars correspond to standard deviations. (C) Hydrolysis of U-mRNA and $m^1\Psi$-mRNA increases the UV absorption of mRNA. This mRNA corresponds to dBroc-mRNA3 in Figure 2C. A_{260} values are normalized to the mean A_{260} values of the non-hydrolysed U-mRNA.

Figure 4: Experimental workflow for the determination of RNA concentration using the mRNACalc webserver. The coloured dots refer to the different RNA hydrolysis methods in Figure 2B.