LEXOGEN
The RNA Experts

SLAMSEQ

SLAMseq Explorer and Kinetics Kits
User Guide

Catalog Numbers:
059 (SLAMseq Explorer Kit - Cell Viability Titration Module)
060 (SLAMseq Explorer Kit - S4U Incorporation Module)
061 (SLAMseq Kinetics Kit - Anabolic Kinetics Module)
062 (SLAMseq Kinetics Kit - Catabolic Kinetics Module)

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For any publication using the SLAMseq kits, please refer to the individual kit modules accordingly as: SLAMseq Explorer Kit - Cell Viability Titration Module, SLAMseq Explorer Kit - S4U Incorporation Module, SLAMseq Kinetics Kit - Anabolic Kinetics Module, and SLAMseq Kinetics Kit - Catabolic Kinetics Module, or refer simply as Lexogen's SLAMseq Kits.

SLAMseq Kits are based on methods developed by the Ameres Group at the Institute of Molecular Biotechnology (IMBA) in Vienna, Austria and should be cited as: Herzog VA, et. al., (2017) Thiol-linked alkylation of RNA to assess expression dynamics. Nature Methods, doi: 10.1038/nmeth. 4435.

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Table of Contents

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1. Overview

This user guide outlines the protocol for Lexogen's SLAMseq Kits, which contain four different Modules:

- The SLAMseq Explorer Kit - Cell Viability Titration Module (Cat. No. 059.24) is required for optimizing reaction conditions for new cell lines.
- The SLAMseq Explorer Kit - S4U Incorporation Module (Cat. No. 060.24) for determining global S4U incorporation before alkylation.
- The SLAMseq Kinetics Kit - Anabolic Kinetics Module (Cat. No. 061.24) measures RNA synthesis kinetics.
- The SLAMseq Kinetics Kit - Catabolic Kinetics Module (Cat. No. 062.24) measures RNA degradation kinetics.

The SLAMseq Kits are used for S4U metabolic labeling and alkylation of RNA, and are intended for use with cultured cells. They are not next generation sequencing (NGS) library prep kits. The SLAMseq Kinetics Kits are designed to be used in conjunction with NGS library preparation for RNA sequencing. Lexogen highly recommends using the QuantSeq 3' mRNA-Seq V2 Library Prep Kits (Cat. No. 191 - 196). Previous versions of QuantSeq 3' mRNA-Seq are also compatible. QuantSeq-Flex Library Prep Modules can be used for targeted RNA sequencing approaches (Cat. No. 028, 166).

Lexogen's SLAMseq (Thiol (SH)-Linked Alkylation for Metabolic Sequencing) kit provides a rapid and scalable method to measure newly synthesized (nascent) and existing RNA levels in parallel. The core SLAMseq workflow involves metabolic labeling of RNA using 4-Thiouridine (S4U) and alkylation of incorporated S4U nucleotides (Fig. 1). In short, cell cultures are incubated with media containing S4U. S4U is taken up by the cells and becomes incorporated into newly synthesized RNA instead of uridine, labeling nascent RNA transcripts. After an alkylation step, total RNA can be used for library preparation. Reverse transcriptase introduces a Guanine (G) instead of an Adenine (A) wherever a modified S4U nucleotide is encountered. Nascent transcripts can therefore be distinguished from existing transcripts in an NGS experiment by mapping to a reference genome and identifying Thymine (T) to Cytosine (C) transitions (T > C conversions).

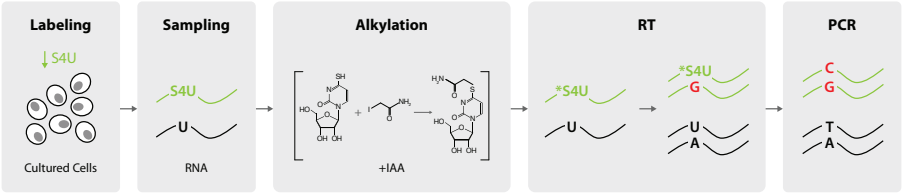


Figure 1. The SLAMseq workflow. Cultured cells are treated with 4-Thiouridine (S4U) for labeling of nascent RNA (green). Total RNA is purified (sampling), and alkylation of the 4-thiol group is induced by the addition of iodoacetamide (IAA). During library preparation, for example using the QuantSeq 3' mRNA-Seq Library Prep Kit, the presence of the resulting carboxyamidomethyl-group causes reverse transcriptase to incorporate guanine (G, in red) instead of adenine (A, in black) at any position where an alkylated *S4U-modified nucleotide is encountered. Second strand synthesis and PCR complete the preparation of a double-stranded library ready for sequencing. In this way, nascent RNA can be distinguished from existing RNA by the presence of T > C mutations in sequencing reads (see Appendix F, p.35).

SLAMseq provides new insights into the control of gene expression. For example, standard RNA sequencing determines steady-state RNA levels only and cannot resolve the underlying kinetics of RNA synthesis and degradation. SLAMseq enhances the resolution of RNA sequencing data by enabling:

- Direct measurement of transcriptional output and nascent RNA concentrations (Anabolic Kinetics Module, Cat. No. 061.24)
- Monitoring RNA turnover and transcript stability (Catabolic Kinetics Module, Cat. No. 062.24).

Before starting a sequencing experiment, we highly recommend testing the optimal concentrations of S4U for your cell line and the time scale of your envisaged experiment by assessing toxicity levels with the SLAMseq Explorer Kit - Cell Viability Titration Module (Cat. No. 059.24), and S4U incorporation rates using the SLAMseq Explorer Kit - S4U Incorporation Module (Cat. No. 060.24), respectively.

ATTENTION: Before starting this protocol, please read the General Guidelines for Lexogen Kits, which are available online. These provide a detailed overview of RNA and kit component handling, as well as general RNA input requirements.

Cell Culture Compatibility

The SLAMseq kits are designed for use with cell suspensions, adherent cells, and 3D-scaffold cell cultures. The seeding, exchange of solutions, and harvesting of cells before RNA purification requires cell culture specific precautions and techniques. The protocol describes these steps using general terms only. Users should be familiar with any specific considerations for the cell culture of interest when applying the SLAMseq protocol. **ATTENTION:** S4U is highly light sensitive and can crosslink. S4U has maximum absorbance at 330 nm (pH 7.5), which extends to 400 nm depending on specific conditions. Therefore, UV and white light are extremely harmful. Keep the cells and all the S4U-containing samples in the dark whenever possible. We recommend switching off the light in the hood, avoiding opening and closing of the incubator during labeling time, shielding samples from white light during incubation times, and work with red light only where possible. If a red light environment is unavailable, plates can be wrapped in foil.

Kit Size

The current SLAMseq kit sizes and formats facilitate initial and small-scale experiments. All examples are given for setting up reactions in a 24-well cell culture plate format using 24× 0.5 - 1 ml growth medium. Alternatively, set-ups are compatible with 12× 2 ml, 48× 0.25 ml, or 96× 0.125 ml plates; or the use of a few small dishes or flasks likewise. The given volumes are provided as a guideline for planning SLAMseq cell culture experiments.
An example RNA kinetics experiment comprising 24 samples would enable testing of two different experimental states (e.g., control vs condition A) sampled at 4 time points in triplicate. Example time points could include e.g., 0, 0.5, 1, and 2 hours to record fast kinetics, or 0, 3, 6, and 12 hours to monitor slow kinetics.

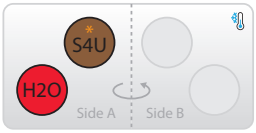
User-Supplied Consumables and Equipment

The kit contains the key components that are specifically required for SLAMseq experiments. All other equipment and consumables, including for cell culture (e.g., PBS, media) and RNA isolation (ethanol, 2-propanol, and TRIzol® Reagent), need to be supplied by the user. Specific requirements are described at the beginning of the respective kit module protocols.

2. Kit Components and Storage Conditions

2.1 SLAMseq Explorer Kit - Cell Viability Titration Module

SLAMseq Explorer Kit - Cell Viability Titration Module, 24 preps (-20 °C)



* Protect from exposure to light!

Figure 2. Location of kit components for the cell viability titration module, Cat. No. 059.24.

Cell Viability Titration Module Cat. No. 059.24 Kit Component	Tube Label	Volume Provided for 24 Preps	Storage
4-Thiouridine (100 mM)	**S4U** ●	900 µl	-20 °C / protect from light!
Molecular Biology Grade Water	**H2O** ●	1,500 µl	-20 °C

ATTENTION: S4U ● is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (e.g., switch off the light in the hood, avoid opening and closing of the incubator during labeling time, shield samples from light during incubation times). Store **S4U** ● at -20 °C and avoid freeze-thaw cycles.

2.2 SLAMseq Explorer Kit - S4U Incorporation Module

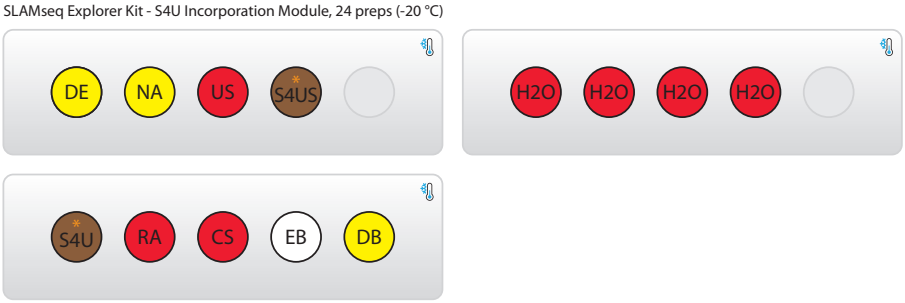
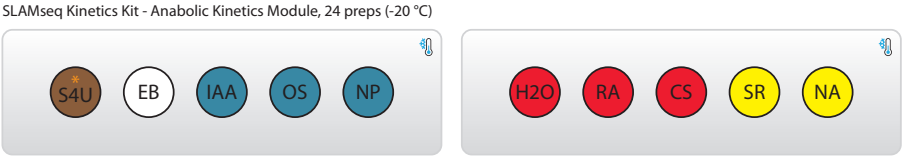


Figure 3. Location of kit components for the S4U incorporation module, Cat. No. 060.24.

S4U Incorporation Module Cat. No. 060.24 Kit Component	Tube Label	Volume Provided for 24 Preps	Storage
4-Thiouridine (100 mM)	**S4U** ●	1,040 µl	-20 °C / protect from light!
Reducing Agent	**RA** ●	1,000 µl	-20 °C
Carrier Substance	**CS** ●	25 µl	-20 °C
Elution Buffer	**EB** ○	1,291 µl	-20 °C
Digestion Buffer	**DB** ●	468 µl	-20 °C
Digestion Enzyme Mix	**DE** ●	53 µl	-20 °C
Sodium Acetate	**NA** ●	159 µl	-20 °C
Molecular Biology Grade Water	**H2O** ●	4x 1,550 µl	-20 °C
Uridine Standard (800 µM)	**US** ●	260 µl	-20 °C
4-Thiouridine Standard (8 µM)	**S4US** ●	300 µl	-20 °C / protect from light!

ATTENTION: S4U ● is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (e.g., switch off the light in the hood, avoid opening and closing of the incubator during labeling time, shield samples from light during incubation times). Store **S4U** ● at -20 °C and avoid freeze-thaw cycles.

2.3 SLAMseq Kinetics Kit - Anabolic Kinetics Module



* Protect from exposure to light!

Figure 4. Location of kit components for the anabolic kinetics module, Cat. No. 061.24.

Anabolic Kinetics Module Cat. No. 061.24 Kit Component	Tube Label	Volume Provided for 24 Preps	Storage
4-Thiouridine (100 mM)	**S4U** ●	1,040 µl	-20 °C / protect from light!
Iodoacetamide 10 mg	**IAA** ●	dissolve in 500 µl 100 % EtOH	-20 °C
Organic Solvent	**OS** ●	704 µl	-20 °C
Sodium Phosphate	**NP** ●	133 µl	-20 °C
Molecular Biology Grade Water	**H2O** ●	1,550 µl	-20 °C
Reducing Agent	**RA** ●	1,000 µl	-20 °C
Carrier Substance	**CS** ●	61 µl	-20 °C
Elution Buffer	**EB** ○	1,291 µl	-20 °C
Stopping Reagent	**SR** ●	31 µl	-20 °C
Sodium Acetate	**NA** ●	159 µl	-20 °C

ATTENTION: S4U ● is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (e.g., switch off the light in the hood, avoid opening and closing of the incubator during labeling time, shield samples from light during incubation times). Store **S4U** ● at -20 °C and avoid freeze-thaw cycles.

2.4 SLAMseq Kinetics Kit - Catabolic Kinetics Module

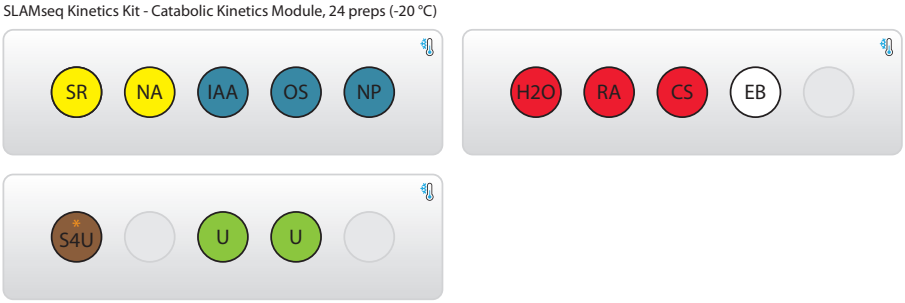


Figure 5. Location of kit components for the catabolic kinetics module, Cat. No. 062.24.

Catabolic Kinetics Module Cat. No. 062.24 Kit Component	Tube Label	Volume Provided for 24 Preps	Storage
4-Thiouridine (100 mM)	**S4U** ●	1,040 µl	-20 °C / protect from light!
Uridine (500 mM)	**U** ●	2x 1,287 µl	-20 °C
Iodoacetamide 10 mg	**IAA** ●	dissolve in 500 µl 100 % EtOH	-20 °C
Organic Solvent	**OS** ●	704 µl	-20 °C
Sodium Phosphate	**NP** ●	133 µl	-20 °C
Molecular Biology Grade Water	**H2O** ●	1,550 µl	-20 °C
Reducing Agent	**RA** ●	1,000 µl	-20 °C
Carrier Substance	**CS** ●	61 µl	-20 °C
Elution Buffer	**EB** ○	1,291 µl	-20 °C
Stopping Reagent	**SR** ●	31 µl	-20 °C
Sodium Acetate	**NA** ●	159 µl	-20 °C

ATTENTION: S4U ● is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (e.g., switch off the light in the hood, avoid opening and closing of the incubator during labeling time, shield samples from light during incubation times). Store **S4U** ● at -20 °C and avoid freeze-thaw cycles.

3. Detailed Protocol

3.1 The SLAMseq Explorer Kit

The Explorer Kit modules are required for optimizing S4U labeling conditions for SLAMseq experiments with cultured cells. Examples are given for experiments in 24-well plate format (see also p.5).

The kit consists of two modules that can be ordered and used individually.

3.1.1 Cell Viability Titration Module

To achieve optimal results in SLAMseq experiments, S4U uptake rates should be maximized without compromising cell viability. S4U cytotoxicity and uptake rates vary between cell types and culture conditions. Therefore, titration of S4U concentrations for metabolic labeling should be performed to determine optimal experimental conditions.

The S4U concentration cytotoxicity is best measured over a time scale that exceeds the labeling duration by a factor of at least 2. For example, use a 12-hour duration when planning 6-hour kinetics experiments.

Cell viability should be evaluated for a titration series of S4U concentrations to generate an inhibition vs S4U concentration curve. Typically, the trace can be fit by a sigmoidal curve to determine the half maximal inhibitory concentration, $IC_{50,ti}$. The experimental working concentration is defined as $IC_{10,ti}$. The $IC_{10,ti}$ level corresponds to the S4U concentration that inhibits 10 % of cells in the time window (ti), which is twice the duration of the intended kinetics experiments (Appendix A, p.28).

The provided reagents enable preparation of 10 ml of cell culture medium for each S4U dilution. The table below outlines the number of replicates (wells) per S4U dilution that can be included using the volume of reagents provided in the Cell Viability Titration Module.

Culture Volume per Well	Maximum No. Wells per Dilution	No. Replicates per Dilution (6 hr)*	No. Replicates per Dilution (12 hr)*
0.2 ml	50	25	12
0.5 ml	20	10	5
1 ml	10	5	2
2 ml	5	2	1
3 ml	3	1	0

*Accounting for 2 media exchanges for 6 hour labeling and 3 media exchanges for 12 hour labeling (see Appendix B, p.30).

Preparation

SLAM-Seq Kit Contents		User-Supplied
S4U H2O	● – thawed at RT*, KEEP IN THE DARK ● – thawed at RT	Cell culture media 50 ml vials Cell viability assay reagents and equipment

* RT = Room Temperature

Measuring S4U Concentration Cytotoxicity

Cells are incubated with a dilution series of 4-Thiouridine (S4U)-containing media in order to determine the optimal concentration for kinetics experiments. Cells should be seeded in culture plates prior to the addition of S4U-containing media. Timing and seeding rates should be adapted for the specified cell type so that optimal confluence is achieved at the end of the intended labeling duration. Cell viability should be measured using an appropriate assay such as the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

ATTENTION: Important notes for Cell Viability Titration Assays!

- **Exchange S4U-containing media every 3 hours.** S4U incorporation rates may decrease over time. Regularly supplying fresh S4U-containing media significantly enhances S4U incorporation rates and allows for a more accurate determination of toxicity measures (see Appendix B, p.30).
- **Protect cell cultures and S4U-containing media from (white) light at all times!** S4U is highly light sensitive and can crosslink. Work under red light and wrap samples with tin foil.

1	Thaw the tube of 4-Thiouridine (**S4U** ●). **REMARK:** Protect the solution from light at all times.
2	Prepare 12 tubes wrapped with tin foil. Add 10 ml of cell culture medium to each tube. Label tubes from 1 to 12.
3	Add 800 µl of the **S4U** ● solution to tube 1 and an additional 9.2 ml cell culture medium, resulting in a total volume of 20 ml. Mix well. **ATTENTION:** Keep the tube wrapped in tin foil to protect it from exposure to white light.
4	Transfer 10 ml of the S4U-containing media from tube 1 into tube 2. Mix well. Tube 2 will now contain 20 ml of a 1:2 dilution of the S4U concentration in tube 1.
5	Transfer 10 ml of S4U-containing media from tube 2 into tube 3. Mix well. Tube 3 will now contain 20 ml of a 1:2 dilution from tube 2 and a 1:4 dilution compared to tube 1.
6	Continue with this 1:2 dilution series as described above until you reach tube number 11.

7	Tube 12 will contain no S4U and will be used for the control cells. Add 800 µl of Molecular Biology Grade Water (**H2O** ●) and an additional 9.2 ml of culture media to tube 12, for a total volume of 20 ml.
8	Pre-warm the S4U-containing media to the desired cell incubation temperature. Remove media from the cells and replace it with the pre-warmed S4U-containing media.
9	Store the 12 tubes at 4 °C between media exchanges. For each media exchange, pre-warm a 2.1 ml aliquot of each S4U dilution in a fresh tube.
10	Exchange the S4U-containing media every 3 hours. The total time should equal twice the labeling duration for intended kinetics experiments, e.g., 12 hours for 6-hour experiments.
11	Measure the cell viability for each S4U concentration using an appropriate cell viability assay (e.g., CellTiter-Glo® Cell Viability Assay (Promega)).
12	Plot the cell viability measure vs concentration to obtain an inhibition vs S4U concentration curve. Tubes 1 to 11 represent a serial 1:2 dilution series, with concentrations ranging from 4 mM to 3.9 µM S4U. Tube 12 is the reference control and contains no S4U. Use this curve to determine the half-maximal inhibitory concentration ($IC_{50,ti}$) and experimental working concentration ($IC_{10,ti}$). A typical result is shown in Appendix A, p.28.

3.1.2 S4U Incorporation Module

Direct validation of S4U incorporation rate is recommended when setting up SLAMseq experiments with new cell types or when experimental conditions are altered (e.g., labeling duration). Global S4U uptake can be measured using a convenient HPLC analysis assay, which comprises four steps. First, cells are cultured in the presence of S4U at the optimal $IC_{10,ti}$ concentration (see Cell Viability Titration Module, p.11). Second, samples are taken at exponentially increasing intervals for a time period equal to twice the labeling duration, e.g., 12 hours for a 6-hour experiment. Third, the RNA is isolated under reducing conditions before being digested to single nucleosides. After precipitation, the samples can be stored at -20 °C. The fourth and final step is HPLC analysis to measure the percentage of incorporated S4U, using two standard curves.

Preparation

SLAM-Seq Kit Contents		User-Supplied
S4U	● – thawed at RT*, KEEP IN THE DARK	Cell culture media
CS	● – thawed at RT	100 % ethanol (EtOH)
RA	● – thawed at RT	TRIzol® Reagent**
EB	○ – thawed at RT	75 % ethanol (EtOH)
DB	● – thawed at RT	Chloroform:isoamyl alcohol mix (24:1)
DE	● – keep on ice or at -20 °C	Acetonitrile
H2O	● – thawed at RT	Triethylamine-acetic acid buffer (TEAA)
NA	● – thawed at RT	Incubator
US	● – thawed at RT	Cell culture plates
S4US	● – thawed at RT, KEEP IN THE DARK	1.5 ml reaction tubes
		SpeedVac
		HPLC columns and equipment

* RT = Room Temperature. ** Caution should be taken when using TRIzol®. Please consult material safety data sheets (MSDS) and use recommended safety procedures for handing and waste disposal.

S4U Labeling of Cultured Cells

Cells are incubated with 4-Thiouridine (**S4U** ●)-containing media. S4U will be incorporated in any newly synthesized RNA transcript instead of uridine. We recommend directly verifying the optimal $IC_{10,ti}$ concentration for the chosen cell type and intended labeling duration, before evaluating incorporation rates (see SLAMseq Explorer Kit - Cell Viability Titration Module, p.11). Reference $IC_{10,ti}$ concentrations are also provided as a guideline for selected cell types (Appendix E, p.34).

ATTENTION: Important notes for S4U Incorporation Assays!

- **Exchange S4U-containing media every 3 hours.** S4U incorporation rates may decrease over time. Regularly supplying fresh S4U-containing media significantly enhances S4U incorporation rates (see Appendix B, p.30).
- **Protect cell cultures and S4U-containing media from (white) light at all times!** S4U is highly light sensitive and can crosslink. Work in the dark or under red light, and wrap samples with tin foil.

1. Seed cells before the labeling experiment to reach maximal confluence or density at the end of the experiment. Seeding rates depend on the respective doubling time.

2. Prepare media containing **S4U** ● at the desired $IC_{10,ti}$ concentration (typically 50 - 500 µM).
REMARK: The concentration depends on the cell type and should be determined beforehand (or see Appendix E, p.34).

3. Remove media from the cells and replace with S4U-containing media.

4. Harvest the cells for RNA sampling at time points of interest. Remove the S4U-containing media and lyse the cells directly in TRIzol®. ☞ Safe stopping point. Samples can be stored at -80 °C at this point.

RNA Isolation - Avoid exposure to light!

Here, a general TRIzol® protocol is described for RNA isolation following S4U labeling. The Reducing Agent (**RA** ●) is important for maintaining the S4U treated samples under constant reducing conditions. Other RNA extraction protocols may be used instead. However, **RA** ● must be added to isolation, wash, and elution buffers (see below).

ATTENTION: Important notes for RNA Isolation!

- It is extremely important to **perform the entire RNA isolation in the dark, or protected from (white) light** exposure (e.g., by keeping the samples covered up, wrapping all tubes with tin foil, or working under red light).
- If other RNA extraction methods are used, **Reducing Agent** (**RA** ●) **must be added** at 1/1,000th of the aqueous volume in isolation and wash buffers, and at 1/100th of the volume in elution or storage buffers.
- If the volume of Reducing Agent (**RA** ●) to add is <1 µl, make a 1:10 dilution of **RA** ● with **H2O** ●.

REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

5. If samples were previously frozen, thaw the lysate and incubate for 5 minutes at room temperature.

6. Add 200 µl chloroform:isoamyl alcohol mix (24:1) per 1 ml of TRIzol® lysate.

7. Shake the tube vigorously for 15 seconds.

8. Incubate for 3 minutes at room temperature.

9. Centrifuge at 16,000 x g for 15 minutes at 4 °C.

10	Transfer the colorless upper aqueous phase to a new tube. Measure the volume of the aqueous phase using a pipette. **ATTENTION:** Careful pipetting is required to avoid transfer of the lower organic phase.
11	Add to the aqueous phase 1 µl of Carrier Substance (**CS** ●), 1/1,000th volumes of Reducing Agent (**RA** ●), and 1 volume of 2-propanol. Vortex well.
12	Incubate for 10 minutes at room temperature.
13	Spin down at 16,000 x g for 20 minutes at 4 °C.
14	Remove and discard the supernatant.
15	Wash the pellet with 500 µl 75 % EtOH and 0.5 µl of Reducing Agent (**RA** ●). Vortex well.
16	Spin down at 7,500 x g for 5 minutes at room temperature.
17	Remove supernatant. Let the pellet dry for 5 - 10 minutes and resuspend it in 20 µl of Elution Buffer (**EB** ○).
18	Incubate for 10 minutes at 55 °C.
19	Measure the concentration by NanoDrop. Proceed with Digestion to Single Nucleosides to prepare the samples for HPLC analysis. ☞ Safe stopping point. At this point RNA can also be stored at -80 °C.

Digestion to Single Nucleosides

By digesting the RNA to single nucleosides and subsequent HPLC analysis, the efficiency of global S4U incorporation can be quantified. This protocol requires µg-scale amounts of total RNA input. DNase I treatment is not required prior to RNA digestion, but can be performed in addition if desired.

REMARK: If HPLC analysis is not feasible, the RNA can be further processed using iodoacetamide for alkylation using the SLAMseq Kinetics Kit Modules (Cat. No. 061, 062). The total RNA after alkylation can then be used as input for NGS library preparation, e.g. with QuantSeq 3′ mRNA-Seq V2 Library Prep Kits (Cat. No. 191 - 196). Previous versions of QuantSeq 3′ mRNA-Seq are also compatible. The S4U incorporation can be evaluated by measuring the frequency of total T > C conversions in comparison to the reference, e.g., by running the sample as a spike-in for a regular single-read NGS run (SR100 read format is recommended).

20	Prepare a mastermix with 18 µl of Digestion Buffer (**DB** ●) and 2 µl of Digestion Enzyme Mix (**DE** ●) per reaction. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.
21	Add 20 µl of the **DB** / **DE** mastermix to a µg-scale amount of isolated total RNA. Bring up the total volume to 130 µl with Molecular Biology Grade Water (**H2O** ●).

22	Incubate overnight (≥16 hours) at 37 °C.
23	Add 6 µl of Sodium Acetate (**NA** ●), 150 µl ice-cold 100 % EtOH, and 3 µl Reducing Agent (**RA** ●). Vortex.
24	Incubate 10 minutes at -80 °C. **REMARK**: Alternatively, incubate on dry ice for 10 minutes, or at -20 °C for 1 hour.
25	Spin down at 12,500 x g for 5 minutes at 4 °C.
26	Transfer the supernatant to a new 1.5 ml tube and discard the pellet.
27	Add 3 µl Reducing Agent (**RA** ●) and 270 µl ice-cold 100 % EtOH to the supernatant. Vortex.
29	Incubate 10 minutes at -80 °C. **REMARK**: Alternatively, incubate on dry ice for 10 minutes, or at -20 °C for 1 hour.
30	Spin down at 12,500 x g for 5 minutes at 4 °C.
31	Transfer the supernatant to a new 1.5 ml tube.
32	Evaporate the supernatant to complete dryness using a vacuum concentrator, e.g., SpeedVac (V-AL setting).
33	Resuspend the sample in 50 µl of Molecular Biology Grade Water (**H2O** ●), and store at -20 °C until the sample is analyzed by HPLC. ☞ Safe stopping point.

HPLC Analysis

Digested RNA samples are compared against dilutions of the Uridine Standard (**US** ●) and S4U Standard (**S4US** ●) using HPLC analysis.

HPLC analysis is carried out using a Supelco Discovery C18 reverse phase (bonded phase 5 µl silica particles) or equivalent column. Mobile phase solutions A and B contain Acetonitrile and Triethylamine-acetic acid buffer (TEAA), and Acetonitrile and Molecular Biology Grade Water (**H2O**), respectively (user-supplied).

34	Take 25 µl of the digested RNA sample and add 75 µl Molecular Biology Grade Water (**H2O** ●).
35	Thaw the Uridine Standard (**US** ●) and S4U Standard (**S4US** ●) tubes. **ATTENTION:** Do not use the **S4U** ● tube!

Prepare 6 standard solutions that contain exponentially increasing concentrations of **US** ● and **S4US** ●.

36

	Volume of			Final Concentration	
	800 µM US ●	8 µM S4US ●	H2O ●	US ●	S4US ●
Std 1	40 µl	50 µl	10 µl	320 µM	4 µM
Std 2	20 µl	25 µl	55 µl	160 µM	2 µM
Std 3	10 µl	12.50 µl	77.50 µl	80 µM	1 µM
Std 4	5 µl	6.25 µl	88.75 µl	40 µM	0.5 µM
Std 5	2.50 µl	3.12 µl	94.38 µl	20 µM	0.25 µM
Std 6	1.25 µl	1.56 µl	97.19 µl	10 µM	0.125 µM

37 Prepare mobile phase solution A with a final concentration of 3 % Acetonitrile, and 0.1 M TEAA, pH 7.0 in H_2O.

38 Prepare mobile phase solution B with a final concentration of 90 % Acetonitrile in H_2O. When using Supelco Discovery C18 reverse phase columns with a size of 250 x 4.6 mm the isocratic gradient to use is: 0 % B for 15 minutes, 0 – 10 % B for 20 minutes, and 10 – 100 % B for 30 minutes. A 5 minute 100 % B wash between the runs is recommended. **REMARK**: These running conditions refer to the method described by ***Spitzer et al., Methods Enzymol. 2014 ; 539: 113–161***, and should be modified with respect to the available column size and type.

39 Prepare the two calibration curves by injecting the standards 1 to 6. Record the chromatogram at 260 nm, and if possible, at 330 nm. The order of retention time is Uridine Standard (**US** ●) followed by S4U Standard (**S4US** ●).

40 Inject the digested RNA samples sequentially and measure the absorbance at Uridine Standard (**US** ●) and S4U Standard (**S4US** ●) retention times at 260 nm and 330 nm. The order of retention times for all ribonucleosides present is: Cytosine, Uridine, Guanine, 4-Thiouridine (S4U), and Adenine. Use the standard curves to define the respective concentrations.

41 To determine the incorporation rate, plot the S4U percentage versus concentration (see Appendix B, p.30).

3.2 The SLAMseq Kinetics Kit

The SLAMseq Kinetics Kit modules are used to measure RNA synthesis and degradation rates by distinguishing nascent from existing RNA as a function of time. The Anabolic Kinetics Module (Cat. No. 061) measures RNA synthesis while the Catabolic Kinetics Module (Cat. No. 062) measures RNA degradation. Each module contains the compounds needed for labeling, stabilizing labeled RNA during isolation, and S4U alkylation. The workflow for S4U RNA labeling differs between Anabolic Kinetics (see 3.2.1) and Catabolic Kinetics (see 3.2.2) experimental designs and is explained in the respective sections.

3.2.1 Anabolic Kinetics Module

The module is optimized for short (pulse) S4U labeling durations. This strategy labels nascent RNA for measuring RNA synthesis rates. Sampling occurs in logarithmic intervals (for example, $2^n \times 15$ minutes). An example of an Anabolic Kinetics labeling experiment result is shown in Appendix C, p.32.

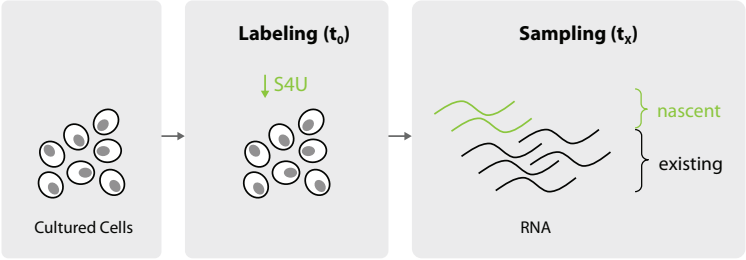


Figure 6. Schematic workflow of SLAMseq for anabolic RNA kinetics measurements. At t_0, modified nucleotides (S4U) are added, which label newly synthesized RNA (nascent, in green). Existing RNA (in black) is unlabeled. At t_x, RNA synthesis is stopped by cell lysis and RNA isolation. Sampling at different intervals, t_x, allows for measurement of transcript synthesis rates.

Preparation

SLAM-Seq Kit Contents		User-Supplied
S4U	● – thawed at RT*, KEEP IN THE DARK	Cell culture media
CS	● – thawed at RT	PBS
RA	● – thawed at RT	100 % ethanol (EtOH)
EB	○ – thawed at RT	2-propanol
IAA	● – thawed at RT	TRIzol® Reagent**
OS	● – thawed at RT	75 % ethanol (EtOH)
NP	● – thawed at RT	Chloroform:isoamyl alcohol mix (24:1)
SR	● – thawed at RT	Incubator
NA	● – thawed at RT	Cell culture plates
H2O	● – thawed at RT	1.5 ml tubes

* RT = Room Temperature. ** Caution should be taken when using TRIzol®. Please consult material safety data sheets (MSDS) and use recommended safety procedures for handing and waste disposal.

S4U Labeling of Cells and Sampling

Cells are incubated with 4-Thiouridine (**S4U** ●)-containing media. S4U will be incorporated into newly synthesized RNA transcripts instead of uridine.

ATTENTION: Important notes for Kinetics Assays!

- **Exchange S4U-containing media every 3 hours.** S4U incorporation rates may decrease over time. Regularly supplying fresh S4U-containing media significantly enhances S4U incorporation rates for longer duration labeling assays (see Appendix B, p.30).
- **Protect cell cultures and S4U-containing media from (white) light at all times!** S4U is highly light sensitive and can crosslink. Work in the dark or under red light, and wrap samples with tin foil.

1. Seed cells before the labeling experiment to reach maximal confluence or density at the end of the experiment. Seeding rates depend on the respective doubling time.
2. Prepare media containing **S4U** ● at the desired $IC_{10,ti}$ concentration (typically 50 - 500 µM).
   REMARK: The concentration depends on the cell type and should be determined beforehand using the SLAMseq Explorer Kit (or see Appendix E, p.34).
3. Remove media from the cells and replace it with S4U-containing media at t_0.
   ATTENTION: S4U is light sensitive. Wrap culture plates in tin foil to prevent exposure to light during incubation.
4. Take off media at desired time points, t_x, and lyse the cells directly in TRIzol®.
   ☞ Safe stopping point. Samples can be stored at -80° C at this point.

RNA Isolation - Avoid exposure to light!

Here, a general TRIzol® protocol is described for RNA isolation following S4U labeling. The Reducing Agent (**RA** ●) is important for maintaining the S4U treated samples under constant reducing conditions. Other RNA extraction protocols may be used instead. However, **RA** ● must be added to isolation, wash and elution buffers (see below).

ATTENTION: Important notes for RNA Isolation!

- It is extremely important to **perform the entire RNA isolation in the dark, or protected from (white) light** exposure (e.g., by keeping the samples covered up, wrapping all tubes with tin foil, or working under red light).
- If other RNA extraction methods are used, **Reducing Agent** (**RA** ●) **must be added** at 1/1,000th of the aqueous volume in isolation and wash buffers, and at 1/100th of the volume in elution or storage buffers.
- If the volume of Reducing Agent (**RA** ●) to add is <1 µl, make a 1:10 dilution of **RA** ● with **H2O** ●.

REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

5	If samples were previously frozen, thaw the lysate and incubate for 5 minutes at room temperature.
6	Add 200 µl chloroform:isoamyl alcohol mix (24:1) per 1 ml of TRIzol® lysate.
7	Shake the tube vigorously for 15 seconds.
8	Incubate for 3 minutes at room temperature.
9	Centrifuge at 16,000 x g for 15 minutes at 4 °C.
10	Transfer the colorless upper aqueous phase to a new tube. Measure the volume of the aqueous phase using a pipette. **ATTENTION:** Careful pipetting is required to avoid transfer of the lower organic phase.
11	Add to the aqueous phase 1 µl of Carrier Substance (**CS** ●), 1/1,000th volumes of Reducing Agent (**RA** ●), and 1 volume of 2-propanol. Vortex well.
12	Incubate for 10 minutes at room temperature.
13	Centrifuge at 16,000 x g for 20 minutes at 4 °C.
14	Remove and discard the supernatant.
15	Wash the pellet with 500 µl 75 % EtOH and 0.5 µl of Reducing Agent (**RA** ●). Vortex well.
16	Centrifuge at 7,500 x g for 5 minutes at 4 °C.
17	Remove supernatant. Let the pellet dry for 5 - 10 minutes, and resuspend it in 16 µl of Elution Buffer (**EB** ○).
18	Incubate for 10 minutes at 55 °C.
19	Measure the concentration by NanoDrop. ☞ Safe stopping point. At this point RNA can be stored at -80 °C. **ATTENTION:** As S4U can become unstable over time it is best to proceed to alkylation with iodoacetamide as soon as possible.
20	Proceed with Iodoacetamide treatment of the RNA to alkylate S4U nucleotides before library preparation and sequencing.

Iodoacetamide Treatment

After total RNA is isolated, the 4-thiol groups present on S4U-labeled transcripts are alkylated with Iodoacteamide (**IAA** ●). When using the resulting modified total RNA for downstream NGS library preparation, such as QuantSeq 3' mRNA-Seq V2 Library preps (Cat. No. 191 - 196), the reverse transcriptase incorporates a Guanine (G) instead of an Adenine (A) wherever an alkylated S4U nucleotide is encountered. Previous versions of QuantSeq 3' mRNA-Seq kits are also compatible.

ATTENTION: The initial steps of the Iodoacetamide treatment with the isolated total RNA **must be performed in the dark, or protected from (white) light exposure** (e.g., by keeping the samples covered up, wrapping all tubes with tin foil, or working under red light).

21 Dissolve 1 tube of Iodoacteamide (**IAA** ●) in 500 µl of 100 % EtOH for a 100 mM final concentration. **ATTENTION:** Use only freshly prepared Iodoacetamide. Test all samples in parallel! Dissolved Iodoacetamide should not be reused.

22 Prepare a mastermix containing 5 µl of the freshly prepared 100 mM Iodoacteamide (**IAA** ●), 25 µl of Organic Solvent (**OS** ●), and 5 µl of Sodium Phosphate (**NP** ●) per sample. **ATTENTION:** **NP** ● can form salt aggregates when added to **OS** ●. This does not affect the downstream reaction, but we recommend to prepare a slightly larger mastermix and transfer just the supernatant to the reaction. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.

23 Mix 15 µl of RNA (up to 5 µg of RNA from step 20) with 35 µl of the **IAA / OS / NP** mastermix. If required, add Molecular Biology Grade Water (**H2O** ●) to a total reaction volume of 50 µl.

24 Incubate the reaction for 15 minutes at 50 °C.

25 Stop the reaction by adding 1 µl of Stopping Reagent (**SR** ●). Mix well. **REMARK:** After this step exposure to light is possible.

26 Add 1 µl of Carrier Substance (**CS** ●), 5 µl of Sodium Acetate (**NA** ●), and 125 µl of 100 % EtOH. Vortex and precipitate for 30 minutes at -80 °C.

27 Centrifuge at 16,000 x g for 30 minutes at 4 °C.

28 Remove the supernatant and wash the pellet with 1 ml 75 % EtOH. Vortex.

29 Centrifuge at 16,000 x g for 10 minutes at 4 °C.

30 Remove the supernatant and let the pellet dry for 5 - 10 minutes.

31 Resuspend in an appropriate volume (5 - 10 µl) of Molecular Biology Grade Water (**H2O** ●).

32 Proceed with RNA quality control and library preparation. For SLAMseq RNA sequencing we recommend using the QuantSeq 3'mRNA-Seq V2 Library Prep Kits (Cat. No. 191 -196).
☞ Safe stopping point. Samples can be stored at -80° C at this point.

3.2.2 Catabolic Kinetics Module

The Catabolic Kinetics Module uses a long S4U labeling step to allow RNA metabolism to reach an approximate steady-state level. The exchange of S4U for unlabeled uridine in the cell culture media stops RNA labeling. Sampling occurs over a time course after unlabeled uridine is added. In this way, RNAs synthesized during the S4U labeling phase represent existing transcripts. Nascent RNAs synthesized after S4U is exchanged for uridine are unlabeled. Measuring the decrease in S4U-labeled existing RNA reveals RNA degradation rates. An example of a Catabolic Kinetics labeling experiment result is shown in Appendix D, p.33.

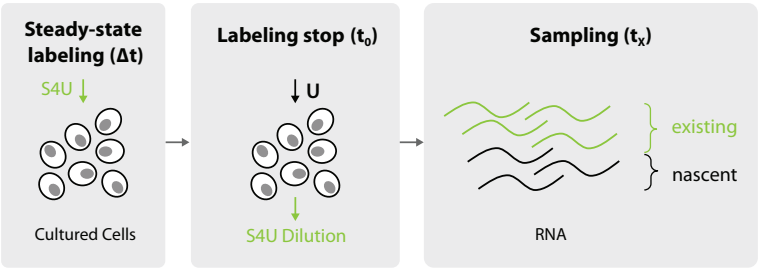


Figure 7. Schematic workflow of SLAMseq for catabolic RNA kinetics measurements. Over Δt, which may extend for up to 24 hours, cells are cultured in S4U-containing media to establish approximate steady-state labeling of the RNA. At t_0, the culture media is replaced with media containing unlabeled uridine (U), which displaces S4U in the cells and stops the labeling of newly synthesized RNA. Subsequently, only existing RNA is labeled (green) while all nascent RNA made after the addition of unlabeled (in black). At t_x, cells are sampled and lysed and RNA is isolated. Sampling at different intervals, t_x, allows for measurement of transcript degradation rates.

Preparation

SLAM-Seq Kit Contents		User-Supplied
S4U	● – thawed at RT*, KEEP IN THE DARK	Cell culture media
U	● – thawed at RT	PBS
CS	● – thawed at RT	100 % ethanol (EtOH)
RA	● – thawed at RT	2-propanol
EB	○ – thawed at RT	TRIzol® Reagent**
IAA	● – thawed at RT	75 % ethanol (EtOH)
OS	● – thawed at RT	Chloroform:isoamyl alcohol mix (24:1)
NP	● – thawed at RT	Incubator
H2O	● – thawed at RT	Cell culture plates
NA	● – thawed at RT	1.5 ml tubes
SR	● – thawed at RT	

* RT = Room Temperature. ** Caution should be taken when using TRIzol®. Please consult material safety data sheets (MSDS) and use recommended safety procedures for handing and waste disposal.

S4U Labeling of Cells

Cells are incubated with 4-Thiouridine (**S4U** ●)-containing media. S4U will be incorporated into newly synthesized RNA transcripts instead of uridine.

ATTENTION: Important notes for Kinetics Assays!

- **Exchange S4U-containing media every 3 hours.** S4U incorporation rates may decrease over time. Regularly supplying fresh S4U-containing media significantly enhances S4U incorporation rates for longer duration labeling assays (see Appendix B, p.30).
- **Protect cell cultures and S4U-containing media from (white) light at all times!** S4U is highly light sensitive and can crosslink. Work in the dark or under red light, and wrap samples with tin foil.

1. Seed cells before the labeling experiment to reach maximal confluence or density at the end of the experiment. Seeding rates depend on the respective doubling time.
2. Prepare media containing **S4U** ● at the desired $IC_{10,ti}$ concentration (typically 50 - 500 µM). **REMARK:** The concentration depends on the cell type and should be determined beforehand (or see Appendix E, p.34).
3. Remove media from the cells and replace it with S4U-containing media at t_0.
4. Incubate cells for up to 24 hours. Exchange media with new S4U-containing media every 3 hours. **ATTENTION:** S4U is light sensitive. Wrap culture plates in tin foil to prevent exposure to light.

Labeling Stop and Sampling

By exchanging the 4-Thiouridine (**S4U** ●) containing media with media containing 100x excess of unlabeled Uridine (**U** ●), the labeling of nascent RNA will be stopped. Newly synthesized transcripts will not contain S4U, while existing transcripts will be labeled with S4U.

ATTENTION: Protect the cells from (white) light during Labeling Stop to prevent cross-linking of S4U already incorporated into the RNA. Wrap culture plates in tin foil to prevent exposure to light, and/or work under red light.

5. Prepare media containing 100x excess of Uridine (**U** ●) relative to the original S4U concentration in the media. **EXAMPLE**: If 100 µM S4U was used during the labeling, the labeling stop media should contain a final concentration of 10 mM **U** ●. Uridine stock concentration is 500 mM. Therefore, for 10 mM, add 480 µl of 500 mM **U** ● to a total volume of 24 ml cell culture medium.
6. Remove the S4U-containing media from the cells at t_0.

7	Wash the cells twice with 1x PBS or cell-compatible cell wash buffer (provided by user).
8	Add the media with excess Uridine (**U** ●) to the cells.
9	Take off media at the time points of interest, t_x, and lyse the cells directly in TRIzol®. ☞ Safe stopping point. Samples can be stored at -80 °C at this point.

RNA Isolation - Avoid exposure to light!

Here, a general TRIzol® protocol is described for RNA isolation following S4U labeling. The Reducing Agent (**RA** ●) is important for maintaining the S4U treated samples under constant reducing conditions. Other RNA extraction protocols may be used instead. However, **RA** ● must be added to isolation, wash, and elution buffers (see below).

ATTENTION: Important notes for RNA Isolation!

- It is extremely important to **carry out the RNA extraction in the dark or protected from (white) light** exposure (e.g., by keeping the samples covered up, wrapping all tubes with tin foil, or working under red light).
- If other RNA extraction methods are used, **Reducing Agent** (**RA** ●) **must be added** at 1/1,000th of the aqueous volume in isolation and wash buffers, and at 1/100th of the volume in elution or storage buffers.
- If the volume of Reducing Agent (**RA** ●) to add is <1 µl, make a 1:10 dilution of **RA** ● with **H2O** ●.

REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

10	If samples were previously frozen, thaw the lysate and incubate for 5 minutes at room temperature.
11	Add 200 µl chloroform:isoamyl alcohol mix (24:1) per 1 ml of TRIzol® lysate.
12	Shake the tube vigorously for 15 seconds.
13	Incubate for 3 minutes at room temperature.
14	Centrifuge at 16,000 x g for 15 minutes at 4 °C.
15	Transfer the colorless upper aqueous phase to a new tube. Measure the volume of the aqueous phase using a pipette. **ATTENTION:** Careful pipetting is required to avoid transfer of the lower organic phase.
16	Add to the aqueous phase 1 µl of Carrier Substance (**CS** ●), 1/1,000th volumes of Reducing Agent (**RA** ●), and 1 volume of 2-propanol. Vortex well.

17	Incubate for 10 minutes at room temperature.
18	Centrifuge 16,000 x g for 20 minutes at 4 °C.
19	Remove and discard the supernatant.
20	Wash the pellet with 500 µl 75 % EtOH and 0.5 µl of Reducing Agent (**RA** ●). Vortex well.
21	Centrifuge at 7,500 x g for 5 minutes at 4 °C.
22	Remove supernatant. Let the pellet dry for 5 - 10 minutes, and resuspend it in 16 µl of Elution Buffer (**EB** ○).
23	Incubate for 10 minutes at 55 °C.
24	Measure the concentration by NanoDrop. ☞ Safe stopping point. At this point RNA can be stored at -80 °C. **ATTENTION:** As S4U can become unstable over time, it is best to proceed to alkylation with iodoacetamide as soon as possible.
25	Proceed with Iodoacetamide treatment of the RNA to alkylate S4U nucleotides before library preparation and sequencing.

Iodoacetamide Treatment

After total RNA is isolated, the 4-thiol groups present on S4U-labeled transcripts are alkylated with Iodoacteamide (**IAA** ●). When using the resulting modified total RNA for downstream NGS library preparation, such as QuantSeq 3' mRNA-Seq V2 Library preps (Cat. No. 191 - 196), the reverse transcriptase incorporates a Guanine (G) instead of an Adenine (A) wherever an alkylated S4U nucleotide is encountered. Previous versions of QuantSeq 3' mRNA-Seq kits are also compatible.

ATTENTION: The initial steps of the Iodoacetamide treatment with the isolated total RNA **must be performed in the dark, or protected from (white) light exposure** (e.g., by keeping the samples covered up, wrapping all tubes with tin foil, or working under red light).

26	Dissolve 1 tube of Iodoacteamide (**IAA** ●) in 500 µl of 100 % EtOH for a 100 mM final concentration. **ATTENTION:** Use only freshly prepared Iodoacetamide. Test all samples in parallel! Dissolved Iodoacetamide should not be reused.
27	Prepare a mastermix containing 5 µl of the freshly prepared 100 mM Iodoacteamide (**IAA** ●), 25 µl of Organic Solvent (**OS** ●), and 5 µl of Sodium Phosphate (**NP** ●) per sample. **ATTENTION: NP** ● can form salt aggregates when added to **OS** ●. This does not affect the downstream reaction, but we recommend to prepare a slightly larger mastermix and transfer just the supernatant to the reaction. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.

28	Mix 15 µl of RNA (up to 5 µg of RNA from step 24) with 35 µl of the **IAA / OS / NP** mastermix. If required, add Molecular Biology Grade Water (**H2O** ●) to a total reaction volume of 50 µl.
29	Incubate the reaction for 15 minutes at 50 °C.
30	Stop the reaction by adding 1 µl of Stopping Reagent (**SR** ●). Mix well. **REMARK:** After this step, exposure to light is possible.
31	Add 1 µl of Carrier Substance (**CS** ●), 5 µl of Sodium Acetate (**NA** ●), and 125 µl of 100 % EtOH. Vortex and precipitate for 30 minutes at -80 °C.
32	Centrifuge at 16,000 x g for 30 minutes at 4 °C.
33	Remove the supernatant and wash the pellet with 1 ml 75 % EtOH. Vortex.
34	Centrifuge at 16,000 x g for 10 minutes at 4 °C.
35	Remove the supernatant and let the pellet dry for 5 - 10 minutes.
36	Resuspend in an appropriate volume (5 - 10 µl) of Molecular Biology Grade Water (**H2O** ●).
37	Proceed with RNA quality control and library preparation. For SLAMseq RNA sequencing, we recommend using the QuantSeq 3′ mRNA-Seq V2 Library Prep Kits (Cat. No. 191 - 196). ☞ Safe stopping point. Samples can be stored at -80° C at this point.

4. Appendix A: Cell Viability Titration Module

S4U uptake varies between cell types and culture conditions. At the start of an experimental series or when using new cell types, the S4U concentration should be titrated to determine optimal experimental conditions for metabolic labeling. The table below outlines the 1:2 dilution series recommended for Cell Viability Titration Assays using this module.

Tube No.	Volume S4U [Tube No.]	Volume Media	S4U Conc. (µM)	S4U Conc. (log_2 µM)
1	800 µl [S4U 100 µM]	19.2 ml	4,000	12.0
2	10 ml [1]	10 ml	2,000	11.0
3	10 ml [2]	10 ml	1,000	10.0
4	10 ml [3]	10 ml	500	9.0
5	10 ml [4]	10 ml	250	8.0
6	10 ml [5]	10 ml	125	7.0
7	10 ml [6]	10 ml	62.5	6.0
8	10 ml [7]	10 ml	31.3	5.0
9	10 ml [8]	10 ml	15.6	4.0
10	10 ml [9]	10 ml	7.8	3.0
11	10 ml [10]	10 ml	3.9	2.0
12	10 ml [11]	10 ml	2.0	1.0

Typical Results

The S4U concentration cytotoxicity is measured over a time scale equal to twice the labeling duration, e.g., 12 hours for 6-hour experiments. The inhibition vs S4U concentration curve is determined by measuring cell viability over an S4U dilution series. Typically, the trace can be fit by a sigmoidal curve to determine the half-maximal inhibitory concentration, $IC_{50,ti}$. The optimal experimental working concentration is defined as the $IC_{10,ti}$: the S4U concentration that would inhibit a maximum of 10 % of cells in the given time window (ti).

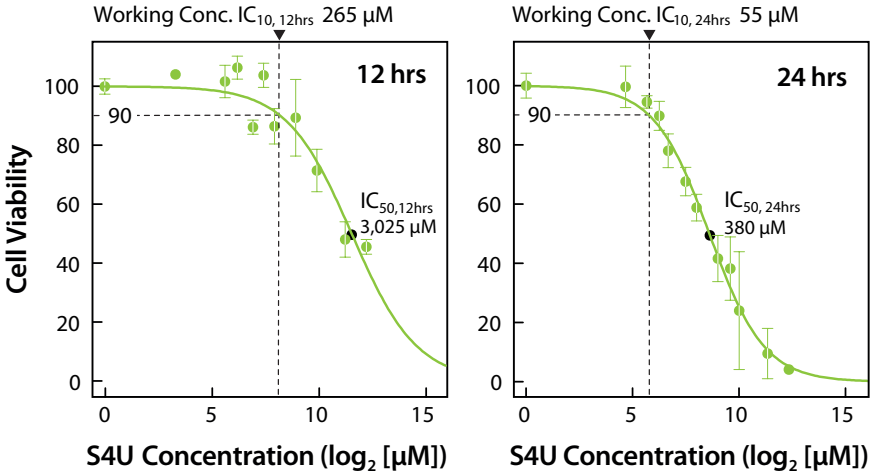


Figure 8. Viability of mouse embryonic stem (mES) cells cultured in the presence of the indicated concentration of 4-Thiouridine (S4U) for 12 hours (left) or 24 hours (right). Viability is expressed relative to untreated cells (100 %). S4U-containing media was exchanged every 3 hours over the course of the labeling experiment. The optimal working concentrations, $IC_{10,ti}$ used in subsequent experiments (265 µM, and 55 µM) are indicated by triangles on top of each plot and dotted lines. Cell viability was measured with the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

5. Appendix B: S4U Incorporation Module

4-Thiouridine (S4U) incorporation rates vary depending on the type of cell line and labeling duration. The S4U Incorporation Module allows for direct measurement of the rate of S4U uptake and incorporation into newly synthesized RNA. Cells are cultured in the presence of S4U-containing media at the pre-determined optimal $IC_{10,ti}$ concentration.

RNA is sampled at time points of exponentially increasing intervals, extending to twice the duration of the intended kinetics experiment (e.g., 0, 4, 8, 12, and 24-hour time points are taken for a 12-hour kinetics experiment). The S4U-containing media is removed at the time points of interest and cells are lysed directly in TRIzol®. Cell lysates can be stored at -80 °C prior to RNA isolation.

After isolation, the RNA is digested to single nucleosides, precipitated, and analyzed by using High Performance Liquid Chromatography (HPLC). The level of S4U incorporated is calculated as a percentage of total uridine for each time point sampled. Plot the incorporation percentage vs time to determine the incorporation rate kinetics.

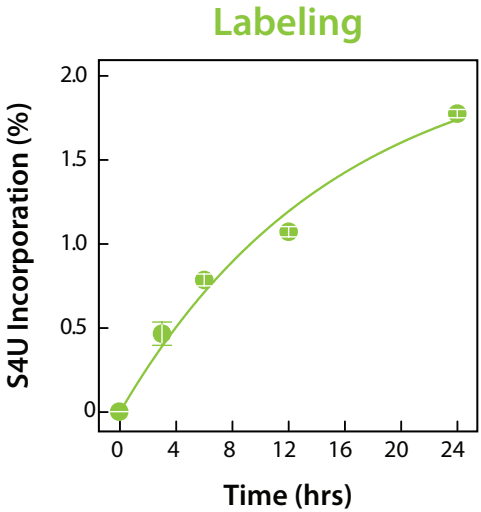


Figure 9. Incorporation rate of S4U as a percentage of total uridine levels, as determined by HPLC. S4U incorporation in total RNA across all time points of a S4U-metabolic labeling experiment in cultured mouse embryonic stem (mES) cells. Values represent mean ± SD of three independent replicates. Maximum incorporation rates after 24 hours of labeling are shown.

REMARK: S4U incorporation rates for mRNA may be higher than estimated by HPLC analysis of single nucleoside-digested total RNA. This is because stable RNA polymerase I and III transcripts, such as rRNA and tRNA, are overrepresented in total RNA but depleted from RNA polymerase II-specific mRNA-Seq libraries.

S4U Media Exchange and S4U Incorporation Rates

S4U incorporation rates may decrease over time, which can affect the level of T > C read counts that will be detected at later timepoints. Regularly supplying fresh S4U-containing media significantly enhances S4U incorporation rates, allows for a more accurate determination of toxicity measures for cell viability assays, and provides more accurate kinetics data for calculating RNA synthesis and degradation rates. Exchanging the S4U-containing media every 3 hours maintains optimal incorporation rates. Longer durations between media exchange may lead to reduced incorporation rates.

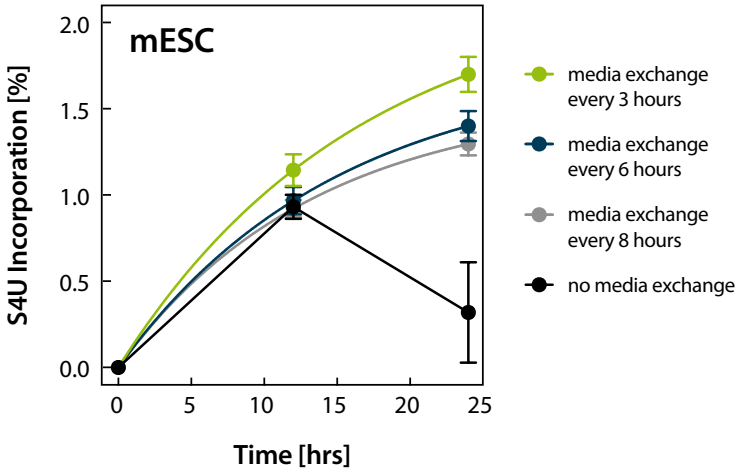


Figure 10. Incorporation rate of S4U for mouse embryonic stem cells (mESC). Media exchange every 3, 6, or 8 hours was compared to no media exchange over a total duration of 24 hours. Cells without media exchange show reduced incorporation rates particularly after 12 - 14 hours. Media exchange every 3 hours produced the highest incorporation rate over time.

Analysis of S4U Incorporation Rate by Sequencing

If HPLC analysis is not feasible, the RNA can be further processed using Iodoacetamide for alkylation using the SLAMseq Kinetics Kit Modules (Cat. No. 061, 062). The total RNA after alkylation can then be used as input for NGS library preparation, i.e., with QuantSeq 3′ mRNA-Seq V2 Library Prep Kits (Cat. No. 191 - 196). Previous versions of QuantSeq 3′ mRNA-Seq are also compatible with SLAMseq. The S4U incorporation can be evaluated by measuring the frequency of total T > C conversions in comparison to the reference, e.g., by running the sample as a spike-in for a regular single-read NGS run (SR100 read format is recommended).

6. Appendix C: Anabolic Kinetics Module

The SLAMseq Kinetics Kit - Anabolic Kinetics differentiates between nascent and existing RNA. At t_0, modified nucleotides (S4U) are added to cell culture media, which results in labeling of newly synthesized RNA. Existing RNA remains unlabeled. At t_x, the RNA synthesis is stopped by cell lysis and RNA isolation. Sampling at different intervals, t_x, allows for measurement of transcript synthesis rates.

Total RNA isolated from SLAMseq anabolic kinetics experiments can be used for NGS library preparation after alkylation with Iodoacetamide. S4U levels in labeled transcripts are distinguished in the final sequencing reads by the presence of T > C nucleotide conversions. Counting the number of reads with T > C conversions over a time course reveals the RNA synthesis kinetics for individual transcripts (see Data Analysis, Appendix F, p.35).

Specific measurement of nascent RNA levels provides insights into transcriptome-wide RNA synthesis dynamics.

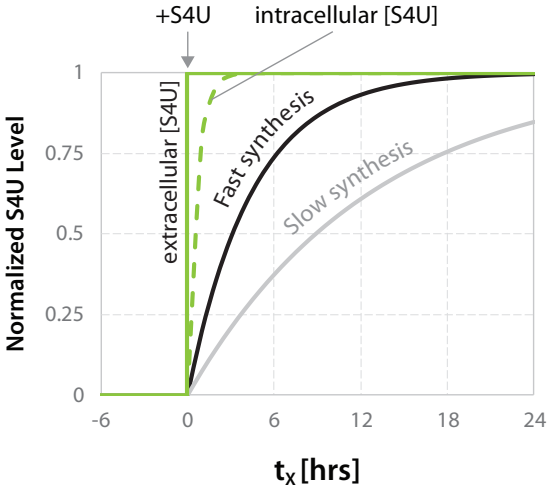


Figure 11. Anabolic kinetics labeling experiment time course. Culturing cells with S4U-containing media (extracellular [S4U], solid green line) changes the intracellular S4U concentration (dashed green line). Nascent RNA will be labelled starting from t_0. Time course measurements determine RNA synthesis rates. Transcripts with fast (black solid line) and slow (gray solid line) synthesis rates can be distinguished by relative differences in the increase in S4U detection over time. S4U levels for individual transcripts are measured by counting sequencing reads with T > C conversions.

7. Appendix D: Catabolic Kinetics Module

The Catabolic Kinetics Module uses a long initial S4U labeling duration to enable RNA metabolism to reach an approximate steady-state level. The exchange of S4U for unlabeled uridine in cell media stops the labeling at t_0. Sampling is carried out over a time course (t_x up to 24 hours) after the unlabeled uridine is added. In this way, existing RNA made during incubation with S4U is labeled, while nascent RNA synthesized after S4U is exchanged for uridine is unlabeled. The experiment monitors RNA degradation rates.

Total RNA isolated from SLAMseq catabolic kinetics experiments can be used for NGS library preparation after alkylation with iodoacetamide. S4U levels in labeled transcripts are distinguished in the final sequencing reads by the presence of T > C nucleotide conversions. Counting the number of reads with T > C conversions over a time course reveals the RNA degradation kinetics for individual transcripts (see Data Analysis, Appendix F, p.35).

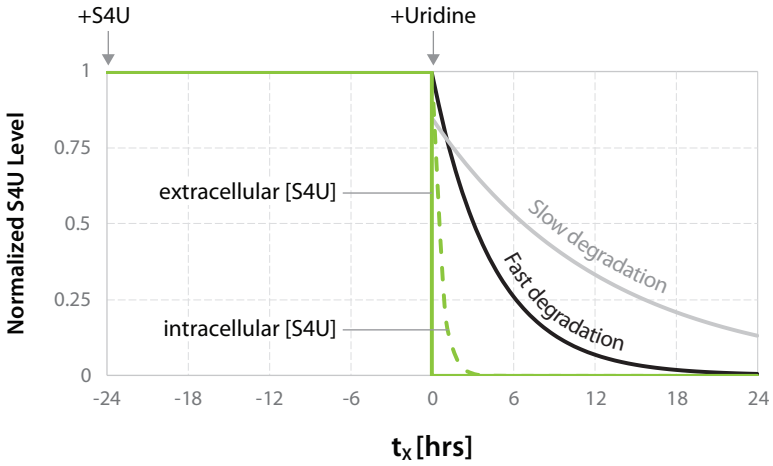


Figure 12. Catabolic kinetics labeling experiment time course. Initial steady-state labeling of RNA is achieved by incubating cells in S4U-containing media for an extended time period, up to 24 hours. The expulsion of S4U from the cells reduces the intracellular S4U concentration back to zero after unlabeled uridine (+Uridine) is added. Only the RNA synthesized before t_0 will be labeled with S4U and levels will decrease as transcripts are degraded over time. Time course measurements taken after unlabeled uridine is added determine fast (solid black line) and slow (solid gray line) degradation rates. S4U levels for individual transcripts are measured by counting sequencing reads with T > C conversions.

8. Appendix E: Cells Tested

Half-maximal inhibitory ($IC_{50,ti}$) and 10 % inhibitory concentrations ($IC_{10,ti}$) have been previously determined for some cell types. The $IC_{10,ti}$ level is considered to be the optimal working S4U concentration and should be determined for a time window (ti) twice the duration of the intended kinetics experiment.

The S4U concentrations in the table below are to be taken as a guideline. These values were measured for time windows of 12 and 24 hours, respectively, using cell viability assays.

Cells tested	$IC_{10,12\ hr}$	$IC_{10,24\ hr}$	$IC_{50,12\ hr}$	$IC_{50,24\ hr}$
Mouse embryonic stem (mES) cells	265 µM	55 µM	3,025 µM	380 µM
K562 myelogenous leukemia cells			2,046 µM	354 µM
MOLT-3 acute lymphoblastic leukemia			2,943 µM	11 µM
MOLM-13 myeloid leukemia cells			53 µM	15 µM
RN2 acute myeloid leukemia cells			50 µM	4 µM
Pseudomonas aeruginosa PAO1 strain	75 µM			

REMARK: We recommend determining $IC_{10,ti}$ concentrations directly for each new cell using the SLAMseq Explorer Kit - Cell Viability Titration Module (Cat. No. 059). Prolonged S4U labeling should always occur at correctly determined $IC_{10,ti}$ S4U concentrations. However, short 1 hour exposure to 100 µM S4U typically shows no effect on cell viability for many cell lines, including: Human Embryonic Kidney Cells (HEK) and Mouse embryonic fibroblasts (MEF), as well as S2, OSC, and Sf9 insect cell lines.

9. Appendix F: Data Analysis

We recommend the use of the SLAMdunk analysis pipeline for analyzing SLAMseq sequencing data, as used in Herzog et al., Thiol-linked alkylation of RNA to assess expression dynamics (Nature Methods, 2017: DOI: 10.1038/nmeth.4435).

For further details, please contact support@lexogen.com.

10. Appendix G: Revision History

Publication No. / Revision Date	Change	Page
059UG142V0105 Dec. 6, 2022	Legal disclaimer updated.	2
	Link to General Guidelines.	5, 6
	Updated Kit Components Figure 2, Figure 3, Figure 4 and Figure 5 and Tables to reflect current packaging and storage requirements.	7 - 10
	Cat. No. updated.	4, 16, 22, 23, 27, 28, 32
059UG142V0104 Sep. 3, 2020	Added SLAMseq logo and associated product list moved to back page.	1, 36
	Updated General terms and conditions.	2
	Grammar changes throughout document.	1-36
059UG142V0100 Oct. 2, 2017	Initial Release.	

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Associated Products:

015 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (FWD))
016 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (REV) with Custom Sequencing Primer)
028 (QuantSeq-Flex Second Strand Synthesis Module for Illumina)
113 - 115, 129 - 131 (QuantSeq 3′ mRNA-Seq Library Prep Kit for Illumina (FWD) with UDI 12nt)
166 (QuantSeq-Flex First Strand Synthesis Module for Illumina V2)
191 - 196 (QuantSeq 3′ mRNA-Seq V2 Library Prep Kit for Illumina (FWD) with UDI 12nt)

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