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)
012 (QuantSeq 3’ mRNA-Seq Library Prep Kit for Ion Torrent)
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)
026 (QuantSeq-Flex First Strand Synthesis Module for Illumina)
028 (QuantSeq-Flex Second Strand Synthesis Module V2 for Illumina)
033 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit with First Strand Synthesis Module)
034 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First Strand Synthesis Module V2)
035 (QuantSeq-Flex Targeted RNA-Seq Library Prep Kit V2 with First and Second Strand Synthesis Modules)
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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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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 not next generation sequencing (NGS) library prep kits. They are used for S4U metabolic labeling and alkylation of RNA and are intended for use with cultured cells. 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 Library Prep Kits (Cat. No. 012, 015, 016). QuantSeq Flex Library Prep V2 Kits can also be used for targeted RNA sequencing approaches (Cat. No. 033, 034, 035).

Lexogen’s SLAMseq (Thiol (SH)-Linked Alkylation for Metabolic Sequencing) 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.

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 a reduced *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.31).
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), and

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.

**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.

**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 duplicate. 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, Cat. No. 059.24

<table>
<thead>
<tr>
<th>Cell Viability Titration Module Cat. No. 059.24 Kit Component</th>
<th>Tube Label</th>
<th>Volume Provided for 24 Preps</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Thiouridine (100 mM)</td>
<td>S4U</td>
<td>900 µl</td>
<td>-20 °C / protect from light!</td>
</tr>
<tr>
<td>RNase-free Water</td>
<td>H₂O</td>
<td>1,500 µl</td>
<td>-20 °C</td>
</tr>
</tbody>
</table>

**ATTENTION:** S4U is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (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, Cat. No. 060.24

![Diagram showing the location of kit components for the S4U incorporation module, Cat. No. 060.24.](image)

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

<table>
<thead>
<tr>
<th>S4U Incorporation Module Cat. No. 060.24</th>
<th>Tube Label</th>
<th>Volume Provided for 24 Preps</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Thiouridine (100 mM)</td>
<td>S4U</td>
<td>1,040 µl</td>
<td>-20 °C / protect from light!</td>
</tr>
<tr>
<td>Reducing Agent</td>
<td>RA</td>
<td>1,000 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Carrier Substance</td>
<td>CS</td>
<td>25 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>EB</td>
<td>1,291 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Digestion Buffer</td>
<td>DB</td>
<td>468 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Digestion Enzyme Mix</td>
<td>DE</td>
<td>53 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>NA</td>
<td>159 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>RNase-free Water</td>
<td>H2O</td>
<td>4x 1,550 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Uridine Standard (800 µM)</td>
<td>US</td>
<td>260 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>4-Thiouridine Standard (8 µM)</td>
<td>S4US</td>
<td>300 µl</td>
<td>-20 °C / protect from light!</td>
</tr>
</tbody>
</table>

**ATTENTION:** S4U ● is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (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, Cat. No. 061.24

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

<table>
<thead>
<tr>
<th>Anabolic Kinetics Module</th>
<th>Tube Label</th>
<th>Volume Provided for 24 Preps</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Thiouridine (100 mM)</td>
<td>S4U</td>
<td>1,040 µl</td>
<td>-20 °C / protect from light!</td>
</tr>
<tr>
<td>Iodoacetamide 10 mg</td>
<td>IAA</td>
<td>dissolve in 500 µl 100 % EtOH</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Organic Solvent</td>
<td>OS</td>
<td>704 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Sodium Phosphate</td>
<td>NP</td>
<td>133 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>RNase-free Water</td>
<td>H₂O</td>
<td>1,550 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Reducing Agent</td>
<td>RA</td>
<td>1,000 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Carrier Substance</td>
<td>CS</td>
<td>61 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>EB</td>
<td>1,291 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Stopping Reagent</td>
<td>SR</td>
<td>31 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>NA</td>
<td>159 µl</td>
<td>-20 °C</td>
</tr>
</tbody>
</table>

**ATTENTION:** S4U is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (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, Cat. No. 062.24

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

<table>
<thead>
<tr>
<th>Catabolic Kinetics Module</th>
<th>Tube Label</th>
<th>Volume Provided for 24 Preps</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Thiouridine (100 mM)</td>
<td>S4U</td>
<td>1,040 µl</td>
<td>-20 °C / protect from light!</td>
</tr>
<tr>
<td>Uridine (500 mM)</td>
<td>U</td>
<td>2x 1,287 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Iodoacetamide 10 mg</td>
<td>IAA</td>
<td>dissolve in 500 µl 100 % EtOH</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Organic Solvent</td>
<td>OS</td>
<td>704 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Sodium Phosphate</td>
<td>NP</td>
<td>133 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>RNase-free Water</td>
<td>H₂O</td>
<td>1,550 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Reducing Agent</td>
<td>RA</td>
<td>1,000 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Carrier Substance</td>
<td>CS</td>
<td>61 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>EB</td>
<td>1,291 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Stopping Reagent</td>
<td>SR</td>
<td>31 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>NA</td>
<td>159 µl</td>
<td>-20 °C</td>
</tr>
</tbody>
</table>

**ATTENTION:** S4U ● is light sensitive and can crosslink. Keep the cells and all the S4U-containing samples in the dark whenever possible (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 optimising S4U labeling conditions for SLAM seq 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 maximised, 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.26).

Preparation

<table>
<thead>
<tr>
<th>SLAM-Seq Kit Contents</th>
<th>User-Supplied</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4U H$_2$O</td>
<td>Cell culture media</td>
</tr>
<tr>
<td></td>
<td>50 ml vials</td>
</tr>
<tr>
<td></td>
<td>Cell viability assay reagents and equipment</td>
</tr>
<tr>
<td></td>
<td>– thawed at RT*, KEEP IN THE DARK</td>
</tr>
<tr>
<td></td>
<td>– thawed at RT</td>
</tr>
</tbody>
</table>

* 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:** S4U incorporation rates may decrease after some hours. Regularly supplying fresh S4U-containing media every 3 hours significantly enhances S4U incorporation, and allows for a more accurate determination of toxicity measures.

**ATTENTION:** S4U is highly light sensitive and can crosslink. Tubes with S4U-containing media and cell cultures must be shielded from light. Working under red light is possible, and wrapping samples with tin foil is recommended.

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 RNase-free 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.
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.

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.

Measure the cell viability for each S4U concentration using an appropriate cell viability assay (e.g., CellTiter-Glo® Cell Viability Assay (Promega)).

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.26.
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 \(\text{IC}_{10,ti}\) concentration (see Cell Viability Titration Module, p.10). 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**

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

* 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 \(\text{IC}_{10,ti}\) concentration for the chosen cell type and intended labeling duration, before evaluating incorporation rates (see SLAMseq Explorer Kit - Cell Viability Titration Module, p.10). Reference \(\text{IC}_{10,ti}\) concentrations are also provided as a guideline for selected cell types (Appendix E, p.30).

**ATTENTION:** S4U incorporation rates may decrease after some hours. Regularly supplying fresh S4U-containing media every 3 hours significantly enhances S4U incorporation consistency.

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 \(\text{IC}_{10,ti}\) concentration (typically 50 - 500 µM).

**REMARK:** The concentration depends on the cell type and should be determined beforehand (or see Appendix E, p.30).
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

It is important to add Reducing Agent (RA) during RNA isolation to maintain the S4U treated sample always under reducing conditions. Here, a general TRIzol® protocol is described. If other RNA extraction methods are used, care should be taken to always add RA 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.

**ATTENTION:** 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. **EXAMPLE:** for 0.5 ml of TRIzol® lysate, add 100 µl of chloroform:isoamyl alcohol mix (24:1).

7. Shake the tube vigorously for 15 seconds.

8. Incubate for 2 - 3 minutes at room temperature.

9. Spin down 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. **EXAMPLE:** If the aqueous phase is 100 µl add 1 µl of CS, 0.1 µl of RA (or 1 µl of 1:10 diluted RA), and 100 µl of 2-propanol.

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.
Wash the pellet with 500 µl 75% EtOH and 0.5 µl of Reducing Agent (RA). Vortex well.

Spin down at 7,500 x g for 5 minutes at room temperature.

Incubate for 2 - 3 minutes at room temperature.

Remove supernatant. Let the pellet dry for 5 - 10 minutes, and resuspend it in 20 µl of Elution Buffer (EB).

Incubate for 10 minutes at 55 °C.

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.

**REMARK:** If HPLC analysis is not feasible, the RNA can be further processed using iodoacetamide for alkylation using the SLAMseq Kinetics Kit - Anabolic Kinetics Module (Cat. No. 061). The total RNA after alkylation can then be used as input for NGS library preparation i.e., with QuantSeq 3’ mRNA-Seq Library Prep Kits (Cat. No. 012, 015, or 016). The S4U incorporation can be evaluated by measuring the frequency of total T to 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).

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.

Add 20 µl of the DB/DE mastermix to a µg-scale amount of isolated total RNA. Make up the total volume to 130 µl with RNase-free Water (H₂O).

Incubate overnight (≥16 hours) at 37 °C.

Add 6 µl of Sodium Acetate (NA), 150 µl ice-cold 100 % EtOH, and 3 µl Reducing Agent (RA). Vortex.

Incubate 10 minutes at -80 °C. **REMARK:** Alternatively, incubate on dry ice for 10 minutes, or at -20 °C for 1 hour.

Spin down at 12,500 x g for 5 minutes at 4 °C.

Transfer the supernatant to a new 1.5 ml tube and discard the pellet.
Add 3 µl Reducing Agent (RA) and 270 µl ice-cold 100 % EtOH to the supernatant. Vortex.

Incubate 10 minutes at -80 °C. REMARK: Alternatively, incubate on dry ice for 10 minutes, or at -20 °C for 1 hour.

Spin down at 12,500 x g for 5 minutes at 4 °C.

Transfer the supernatant to a new 1.5 ml tube.

Evaporate the supernatant to complete dryness using a vacuum concentrator, e.g., SpeedVac (V-AL setting).

Resuspend the sample in 50 µl of RNase-free 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 RNase-free Water (H₂O), respectively (user-supplied).

Take 25 µl of the digested RNA sample and add 75 µl RNase-free Water (H₂O).

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.

<table>
<thead>
<tr>
<th>Std</th>
<th>Volume of US</th>
<th>Volume of S4US</th>
<th>Volume of H₂O</th>
<th>Volume of Final Concentration US</th>
<th>Volume of Final Concentration S4US</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std 1</td>
<td>40 µl</td>
<td>50 µl</td>
<td>10 µl</td>
<td>320 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td>Std 2</td>
<td>20 µl</td>
<td>25 µl</td>
<td>55 µl</td>
<td>160 µM</td>
<td>1.6 µM</td>
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<tr>
<td>Std 3</td>
<td>10 µl</td>
<td>12.50 µl</td>
<td>77.50 µl</td>
<td>80 µM</td>
<td>0.8 µM</td>
</tr>
<tr>
<td>Std 4</td>
<td>5 µl</td>
<td>6.25 µl</td>
<td>88.75 µl</td>
<td>40 µM</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Std 5</td>
<td>2.50 µl</td>
<td>3.12 µl</td>
<td>94.38 µl</td>
<td>20 µM</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Std 6</td>
<td>1.25 µl</td>
<td>1.56 µl</td>
<td>97.19 µl</td>
<td>10 µM</td>
<td>0.1 µM</td>
</tr>
</tbody>
</table>

Prepare mobile phase solution A with a final concentration of 3 % Acetonitrile, and 0.1 M TEAA, pH 7.0 in H₂O.
Prepare mobile phase solution B with a final concentration of 90 % Acetonitrile in H₂O. When using Supelco Discovery C18 reverse phase columns with a size of 250 x 46 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.

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).

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.

To determine the incorporation rate, plot the S4U percentage versus concentration (see Appendix B, p.27).
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 and 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 of $2^n \times 15$ minutes. An example of an Anabolic Kinetics labeling experiment result is shown in Appendix C, p.28.

![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.](image)

**Preparation**

<table>
<thead>
<tr>
<th>SLAM-Seq Kit Contents</th>
<th>User-Supplied</th>
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</thead>
<tbody>
<tr>
<td>S4U</td>
<td>– thawed at RT*, <strong>KEEP IN THE DARK</strong></td>
</tr>
<tr>
<td>CS</td>
<td>– thawed at RT</td>
</tr>
<tr>
<td>RA</td>
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</tr>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>Cell culture media</td>
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</tr>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>100 % ethanol (EtOH)</td>
<td></td>
</tr>
<tr>
<td>2-propanol</td>
<td></td>
</tr>
<tr>
<td>TRizol® Reagent**</td>
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</tr>
<tr>
<td>75 % ethanol (EtOH)</td>
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</tr>
<tr>
<td>Chloroform:isoamyl alcohol mix (24:1)</td>
<td></td>
</tr>
<tr>
<td>Incubator</td>
<td></td>
</tr>
<tr>
<td>Cell culture plates</td>
<td></td>
</tr>
<tr>
<td>1.5 ml tubes</td>
<td></td>
</tr>
<tr>
<td>SpeedVac</td>
<td></td>
</tr>
</tbody>
</table>

* RT = Room Temperature. ** Caution should be taken when using TRizol®. Please consult material safety data sheets (MSDS) and use recommended safety procedures for handling 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:** In some cell media the effective S4U concentration may decrease after a few hours. Supplying fresh S4U-containing media every 3 hours significantly enhances S4U incorporation.

1. Seed cells the 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$_{10.0}$ concentration (typically 50 - 500 µM).

   **REMARK:** The concentration depends on the cell type and should be determined beforehand (or see Appendix E, p.30).

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

It is important to add Reducing Agent (RA) during RNA isolation to maintain the S4U treated sample always under reducing conditions. Here, a general TRIzol® protocol is described. If other RNA extraction methods are used, care should be taken to always add Reducing Agent (RA) 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.

**ATTENTION:** If the volume of Reducing Agent (RA) to add is < 1 µl, make a 1:10 dilution of RA with H$_2$O. **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. **EXAMPLE:** for 0.5 ml of TRIzol® lysate, add 100 µl of chloroform:isoamyl alcohol mix (24:1).

7. Shake the tube vigorously for 15 seconds.

8. Incubate for 2 - 3 minutes at room temperature.

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

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. **EXAMPLE:** If the aqueous phase is 100 µl add 1 µl of CS, 0.1 µl of RA (or 1 µl of 1:10 diluted RA), and 100 µl of 2-propanol.

---

**Iodoacetamide Treatment**

After total RNA is isolated, the 4-thiol groups present on S4U-labeled transcripts are alkylated with Iodoacetamide (IAA). When using the resulting modified total RNA for downstream NGS library preparation, such as QuantSeq 3’ mRNA-Seq Library preps (Cat. No. 012, 015, or 016), the reverse transcriptase incorporates a Guanine (G) instead of an Adenine (A) wherever an alkylated S4U nucleotide is encountered.

Dissolve 1 tube of Iodoacetamide (IAA) in 500 µl of 100% EtOH for a 100 mM final concentration. **ATTENTION:** Use only freshly prepared Iodoacetamide. Test all samples in parallel!
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). 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.

Mix up to 5 µg of RNA (from step 20) with the IAA / OS / NP mastermix. Make up the total reaction volume to 50 µl with RNase-free water (H₂O).

Incubate the reaction at 50 °C for 15 minutes.

Stop the reaction by adding 1 µl of Stopping Reagent (SR).

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.

Spin down at 16,000 x g for 30 minutes.

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

Spin down at 16,000 x g for 10 minutes.

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

Resuspend in an appropriate volume (5 - 10 µl) of RNase-free water (H₂O).

Proceed with RNA quality control and library preparation. For SLAMseq RNA sequencing we recommend using the QuantSeq 3’ mRNA-Seq Library Prep Kits (Cat. No. 012, 015, or 016).
### 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 in 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.29.

![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 t₀, 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 tₓ, cells are sampled and lysed and RNA is isolated. Sampling at different intervals, tₓ, allows for measurement of transcript degradation rates.](image)

#### Preparation

<table>
<thead>
<tr>
<th>SLAM-Seq Kit Contents</th>
<th>User-Supplied</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4U</td>
<td>Cell culture media</td>
</tr>
<tr>
<td>U</td>
<td>PBS</td>
</tr>
<tr>
<td>CS</td>
<td>100 % ethanol (EtOH)</td>
</tr>
<tr>
<td>RA</td>
<td>2-propanol</td>
</tr>
<tr>
<td>EB</td>
<td>TRIzol® Reagent**</td>
</tr>
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<td>IAA</td>
<td>75 % ethanol (EtOH)</td>
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<td>OS</td>
<td>Chloroform:isoamyl alcohol mix (24:1)</td>
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<td>Incubator</td>
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<tr>
<td>H₂O</td>
<td>Cell culture plates</td>
</tr>
<tr>
<td>NÅ</td>
<td>1.5 ml tubes</td>
</tr>
<tr>
<td>SR</td>
<td>SpeedVac</td>
</tr>
</tbody>
</table>

* 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:** In some cell media the effective S4U concentration may decrease after few hours. Supplying fresh S4U-containing media every 3 hours significantly enhances S4U incorporation.
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.

Prepare media containing S4U at the desired IC\textsubscript{10\%} concentration (typically 50 - 500 µM).

**REMARK:** The concentration depends on the cell type and should be determined beforehand (or see Appendix E, p.30).

Remove media from the cells and replace it with S4U-containing media at t\textsubscript{0}.

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.

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.

Remove the S4U-containing media from the cells at t\textsubscript{0}.

Wash the cells twice with 1x PBS or cell-compatible cell wash buffer (provided by user).

Add the media with excess Uridine (U) to the cells.

Take off media at the time points of interest, t\textsubscript{x}, and lyse the cells directly in TRIzol®.

**RNA Isolation**

It is important to add Reducing Agent (RA) during RNA isolation to maintain the S4U treated sample always under reducing conditions. Here, a general TRIzol® protocol is described. If other RNA extraction methods are used, care should be taken to always add RA at 1/1,000\textsuperscript{th} of the aqueous volume in isolation and wash buffers, and at 1/100\textsuperscript{th} of the volume in elution or storage buffers.

**ATTENTION:** If the volume of Reducing Agent (RA) to add is < 1 µl, make a 1:10 dilution of RA with H\textsubscript{2}O. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.
If samples were previously frozen, thaw the lysate and incubate for 5 minutes at room temperature.

Add 200 µl chloroform:isoamyl alcohol mix (24:1) per 1 ml of TRIzol® lysate. **EXAMPLE:** for 0.5 ml of TRIzol® lysate, add 100 µl of chloroform:isoamyl alcohol mix (24:1).

Shake the tube vigorously for 15 seconds.

Incubate for 2 - 3 minutes at room temperature.

Spin down at 16,000 x g for 15 minutes at 4 °C.

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.

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. **EXAMPLE:** If the aqueous phase is 100 µl add 1 µl of **CS**, 0.1 µl of **RA** (or 1 µl of 1:10 diluted **RA**), and 100 µl of 2-propanol.

Incubate for 10 minutes at room temperature.

Spin down at 16,000 x g for 20 minutes at 4 °C.

Remove and discard the supernatant.

Wash the pellet with 500 µl 75 % EtOH and 0.5 µl of Reducing Agent (**RA**). Vortex well.

Spin down at 7,500 x g for 5 minutes at room temperature.

Incubate for 2 - 3 minutes at room temperature.

Remove supernatant. Let the pellet dry for 5 - 10 minutes, and resuspend it in 20 µl of Elution Buffer (**EB**).

Incubate for 10 minutes at 55 °C.

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.**

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 iodoacetamide (IAA). When using the resulting modified total RNA for downstream NGS library preparation, such as QuantSeq 3’ mRNA-Seq Library preps (Cat. No. 012, 015, or 016), the reverse transcriptase incorporates a Guanine (G) instead of an Adenine (A) wherever an alkylated S4U nucleotide is encountered.

1. **Dissolve 1 tube of iodoacetamide (IAA) in 500 µl of 100 % EtOH for a 100 mM final concentration.** **ATTENTION:** Use only freshly prepared iodoacetamide. Test all samples in parallel!

2. **Prepare a mastermix containing 5 µl of the freshly prepared 100 mM iodoacetamide (IAA), 25 µl of Organic Solvent (OS), and 5 µl of Sodium Phosphate (NP).** **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.

3. **Mix up to 5 µg of RNA (from step 25) with the IAA / OS / NP mastermix.** Make up the total reaction volume to 50 µl with RNase-free water (H₂O).

4. **Incubate the reaction at 50 °C for 15 minutes.**

5. **Stop the reaction by adding 1 µl of Stopping Reagent (SR).**

6. **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.**

7. **Spin down at 16,000 x g for 30 minutes.**

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

9. **Spin down at 16,000 x g for 10 minutes.**

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

11. **Resuspend in an appropriate volume (5 - 10 µl) of RNase-free water (H₂O).**

12. **Proceed with RNA quality control and library preparation.** For SLAMseq RNA sequencing we recommend using the QuantSeq 3’ mRNA-Seq Library Prep Kits (Cat. No. 012, 015, or 016).
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 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$_{50}$. The optimal experimental working concentration is defined as the IC$_{10}$: the S4U concentration that would inhibit a maximum of 10% of cells in the given time window (ti).

![Graph showing cell viability as a function of S4U concentration.](image)

**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$_{10}$, 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$_{10,01}$ 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.
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 to C nucleotide conversions. Counting the number of reads with T to C conversions over a time course reveals the RNA synthesis kinetics for individual transcripts (see Data Analysis, Appendix F, p.31).

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

![Figure 10. 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 (grey 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 to C conversions.](image-url)
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 to C nucleotide conversions. Counting the number of reads with T to C conversions over a time course reveals the RNA degradation kinetics for individual transcripts (see Data Analysis, Appendix F, p.31).

![Figure 11. Catabolic kinetics labeling experiment time course.](image)

Figure 11. 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 grey line) degradation rates. S4U levels for individual transcripts are measured by counting sequencing reads with T to C conversions.
8. Appendix E: Cells Tested

Half-maximal inhibitory (IC\textsubscript{50,ti}), and 10 % inhibitory concentrations (IC\textsubscript{10,ti}) have been previously determined for some cell types. The IC\textsubscript{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.

<table>
<thead>
<tr>
<th>Cells tested</th>
<th>IC\textsubscript{10,12 h}</th>
<th>IC\textsubscript{10,24 h}</th>
<th>IC\textsubscript{50,12 h}</th>
<th>IC\textsubscript{50,24 h}</th>
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<tbody>
<tr>
<td>Mouse embryonic stem (mES) cells</td>
<td>265 µM</td>
<td>55 µM</td>
<td>3,025 µM</td>
<td>380 µM</td>
</tr>
<tr>
<td>K562 myelogenous leukemia cells</td>
<td></td>
<td></td>
<td>2,046 µM</td>
<td>354 µM</td>
</tr>
<tr>
<td>MOLT-3 acute lymphoblastic leukemia</td>
<td></td>
<td></td>
<td>2,943 µM</td>
<td>11 µM</td>
</tr>
<tr>
<td>MOLM-13 myeloid leukemia cells</td>
<td></td>
<td></td>
<td>53 µM</td>
<td>15 µM</td>
</tr>
<tr>
<td>RN2 acute myeloid leukemia cells</td>
<td></td>
<td></td>
<td>50 µM</td>
<td>4 µM</td>
</tr>
</tbody>
</table>

REMARK: We recommend determining IC\textsubscript{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\textsubscript{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 SLAM-DUNK 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).

This modified alignment algorithm is specially adapted to report read counts for T to C (T>C) containing reads for downstream analysis. For further details of SLAMseq data analysis, or enquiries regarding use of SLAM-DUNK for commercial use please contact info@lexogen.com.

10. Appendix G: Revision History

<table>
<thead>
<tr>
<th>Publication No./Revision date</th>
<th>Change</th>
<th>Page</th>
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<td>059UG142V0101 Oct. 3, 2017</td>
<td>Kit component layout modified, minor consistency changes.</td>
<td>6, 8, 9</td>
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</table>
SLAM-Seq Explorer and Kinetics Kits · User Guide

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