LEXOGEN

Enabling complete transcriptome sequencing

AUTO™ SENSE

Making sense of HT RNA Sequencing

mRNA-Seq Library Prep Kit V2
on the PerkinElmer Sciclone/Zephyr NGS Workstations

User Guide

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001.24 (SENSE mRNA-Seq Library Prep Kit V2 for Illumina, including Barcode Set 1 – 3, 24 preps)
001.96 (SENSE mRNA-Seq Library Prep Kit V2 for Illumina, including Barcode Set 1 – 12, 96 preps)
024.96 (Automation Module for SENSE mRNA-Seq V2, 96 preps)

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1. Introduction

The autoSENSE mRNA-Seq Kit V2 is an automated all-in-one library preparation protocol designed to generate Illumina-compatible libraries from total RNA within nine hours. It uses the reagents of the SENSE mRNA-Seq kit V2 and, optionally, the Automation Module for SENSE mRNA Seq V2, and runs on the Perkin-Elmer Sciclone NGS and Zephyr NGS liquid handlers. If not specified otherwise, the properties of the SENSE protocol also apply to autoSENSE V2.

The SENSE protocol maintains strand-specificity (> 99.9 %) and allows the mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. SENSE includes an integrated poly(A) selection, so prior rRNA depletion is not required. Insert size can be varied during the library preparation protocol itself, meaning that size selection with additional kits is not necessary. Optional multiplexing of libraries is carried out using up to 96 external barcodes. Libraries are compatible with both single-end and paired-end sequencing reagents.

The SENSE protocol comprises a highly specific bead-based poly(A) selection step which removes almost all traces of rRNA, tRNA, and non-polyadenylated RNA. Information regarding input RNA requirements can be found in Appendix B (p.29).

Library production is initiated by the random hybridization of starter/stopper heterodimers to the poly(A) RNA still bound to the magnetic beads. These starter/stopper heterodimers contain Illumina-compatible linker sequences. A single-well reverse transcription and ligation reaction extends the starter to the next hybridized heterodimer, where the newly-synthesized cDNA insert is ligated to the stopper. As the insert size is determined by the distance between starter/stopper binding sites RNA fragmentation is not required. Therefore, spurious second strand synthesis from the 5' ends of fragments is absent, providing the basis for the excellent strand-specificity of the autoSENSE V2 protocol.

Second strand synthesis is performed to release the library from the oligodT beads, and the library is then purified using magnetic beads. In a subsequent PCR amplification the complete sequences required for cluster generation are introduced. Library quantification can be performed with standard protocols and is further discussed in Appendix D (p.34). Libraries are compatible with single-end or paired-end sequencing. External barcodes are included in the kit and are also introduced during the PCR amplification step (Appendix E, p.36). Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of the SENSE data, such as read orientation, are presented in Appendix G (p.37).

Automating the process of library preparation has the advantage of avoiding sample tracking errors and reducing sample-to-sample variability while dramatically increasing throughput. autoSENSE V2 is running on the liquid handler platforms by PerkinElmer. In the protocol described in

this user guide, the prePCR phase is programmed for the Sciclone NGS Workstation whereas the postPCR phase (cleanup) is programmed for the Zephyr NGS Workstation. However, the post-PCR phase can also be implemented on a Sciclone NGS workstation (ask Lexogen for details). Running prePCR and postPCR processing on separate machines, potentially in separate rooms, substantially reduces the risk of cross-contamination of the prePCR samples by PCR products. The whole process runs with a single manual relocation of the sample plate to and from an external device (Phase 2–PCR Amplification on a 96-well thermocycler). Phase1–PrePCR can run without any operator intervention or supervision, if the reaction wells are sealed with oil (oil is included in the Automation Module for SENSE mRNA-Seq V2), during second strand synthesis thermal cycle. Optionally, if the sealing is done with a film, manual intervention is required for sealing and unsealing before and after the thermal cycle, respectively. Phase 3–PostPCR needs a single manual intervention (plate removal).

The autoSENSE V2 is typically used for preparing 96 barcoded libraries. The set of 96 barcodes is included in the 96 prep kit in a 96-well barcode plate. The liquid handler program allows for processing of samples in multiples of 8 reactions (full columns of a 96-well plate) up to and including 96. If fewer than 96 samples are to be processed, the barcodes can be selectively transferred from the kit barcode plate to a user-specific barcode plate. A single kit can be used for several machine runs until all the barcodes are used up.

The setup of the machine run involves preparation of simple master mixes from the kit components, aliquoting the reagents to the respective plates, and placing the plates on deck. A Microsoft Excel workbook (included in the software distribution part of the kit) contains easy-to-follow preparation guidelines and recipes for master mix preparations. The workbook automatically adjusts the recipes to the number of samples processed.

Two different workflow options are available for bead washing at the beginning of the protocol. The beads can either be washed automatically by the liquid handler, or they can be washed manually in one or several larger batches. The latter option reduces the machine time. This is typically preferred for high-throughput sample processing with several machine runs in a series.

The whole autoSENSE V2 protocol (96 samples) can be run in nine hours in total, including about two hours of manual setup time. Since the individual protocol phases run on separate machines, further throughput enhancement can be achieved by parallelizing the workflow.

Running an installed protocol is a simple laboratory task which only requires standard skills and know-how covered by the basic introductory training, which is part of the liquid handler installation. Installation of a new protocol on the liquid handler is, however, a task requiring certain additional skills and experience. Ask Lexogen for installation support as well as for any user-specific kit customization, such as different barcode-to-well allocation, different kit sizing, or similar.

2. autoSENSE mRNA-Seq V2 Library Prep Workflow Overview

Phase 1–PrePCR on the Sciclone NGS Workstation

All reagents are prefilled in the plates on the deck. The plates containing RNA samples, enzymes, and some other reagents are placed on thermolocators[1] held at +10 °C.

Beads Preparation

The magnetic oligodT beads (MBR) are supplied in a viscous storage buffer and need to be washed thoroughly before use. This can either be done automatically by the workstation for each single reaction separately, or manually in a larger batch (one of the two options needs to be selected in the workbook). The beads are first aliquoted to the respective wells. The washing involves separating the beads on a magnet, removing the storage buffer as supernatant, then resuspending the beads in 100 µl Bead Wash Buffer (BW) off the magnet, separating on the magnet and removing the supernatant again. The washing step is repeated two times. The beads are resuspended in 10 µl Hybridization Buffer (HYB).

RNA Denaturation and Hybridization

RNA samples are briefly heated on a thermolocator at 60 °C for 1 minute to resolve secondary structures and promote efficient hybridization. The washed oligodT beads in Hybridization Buffer are then added to the RNA samples and incubated on a thermoshaker at 25 °C for 20 minutes with 500 rpm agitation. During this incubation the Starter/Stopper/Reverse Transcription and Ligation Mix (RTST, prepared manually from RTL or RTS, depending on the required library length, and ST) and the Reverse Transcription and Ligation/Enzyme Mix (RTE1, prepared manually from RTL or RTS and E1) are aliquoted (prearrayed) into a 384-well plate for later use. After separation on the magnet the supernatant is removed and the beads are washed again with 100 µl Bead Wash Buffer (BW). The beads are incubated on the thermoshaker at 25 °C for 5 minutes with 800 rpm agitation, separated on the magnet and the supernatant is removed. The washing step is repeated once again for a total of two washing steps.

After this step, each used well of the plate contains non-resuspended beads with bound polyadenylated RNA.

Reverse Transcription and Ligation

The prearrayed Starter/Stopper/Reverse Transcription and Ligation Mix (RTST, 15 µl) is added to the beads, followed by mixing on thermoshaker at 25 °C for 5 minutes with 1250 rpm agitation. If the RNA input material is less than 10 ng, the mixing is extended to 20 minutes (this can be

[1] Thermolocator is a heating/cooling unit positioned at a specific location of the deck.

specified in the workbook). Then 5 µl of Reverse Transcription and Ligation/Enzyme Mix (RTE1) are added to the beads. The beads are resuspended and the mix is incubated on a thermoshaker at 25 °C for 2 minutes with 1250 rpm agitation. The temperature of the thermoshaker is then raised to 37 °C, and the ligation takes place during a 1 hour incubation with 1250 rpm agitation. The reaction mix is freed from all unbound components by two consecutive bead wash steps, using 100 µl of the Bead Wash Buffer (BW) each. The beads are separated on the magnet, and the supernatant is removed.

After this step each used well of the plate contains non-resuspended beads with starter/stopper heterodimers hybridized to the RNA. The reverse transcription and ligation have been performed, so short cDNA fragments with linker sequences at either end are generated.

Second Strand Synthesis

The beads are resuspended in 20 µl Second Strand Synthesis/Enzyme Mix (SE, prepared manually of E2 and SSM). 20 µl of Silicon Oil (OIL) are gently pipetted on top of the liquid as a sealing for the subsequent thermal cycle (OIL is included in the Automation Module for SENSE mRNA-Seq V2). Alternatively, manual film sealing can be used. In this case, the liquid handler stops and the operator is asked to take the plate, seal it with film, put it back on deck, and resume run. Using oil (without operator intervention during the run) or film (with operator intervention) can be specified in the workbook. In the meantime, one of the thermolocators has been preheated to 98 °C, the other one to 65 °C. The plate is placed on the 98 °C thermolocator, stays there for 90 seconds, then it is placed on the 65 °C thermolocator. After 60 seconds, the temperature is raised to 72 °C, and the plate is kept there for another 5 minutes. If film is used for sealing, the liquid handler stops again, and the operator is asked to take the plate, spin it down shortly, remove the sealing film, place the plate back on deck, and resume the run. The plate is then placed on a magnet.

After this step each well in use contains double-stranded cDNA freed from the RNA still hybridized to beads and partially hydrolyzed. Each well contains 20 µl of the cDNA/beads solution (with 20 µl of Silicon Oil on top if oil has been used for sealing).

Purification after Second Strand Synthesis

With the plate on the magnet, the samples (optionally including oil) are gently transferred to new, clean wells. A double purification step follows: Purification Beads (PB) diluted with purification solutions (PS) and, optionally, Bead Diluent (BD), 16 µl total volume (the particular mixing ratio of PB, PS, and BD determines the final library length and can be specified in the workbook, see Appendix C, p.31), are dispensed to the used sample wells and incubated on a thermoshaker at 20 °C for 10 minutes at 500 rpm agitation (BD is included in the Automation Module for SENSE mRNA Seq V2). The plate is then placed on the magnet, the beads are separated, and the supernatant (56 µl for oil sealing, 36 µl for film sealing) is removed. Each well in use is then washed with 100 µl Ethanol (80%) on the magnet. The Ethanol is removed after 30 seconds of incubation, and the Ethanol wash step is repeated once again. The beads are drying for 5 minutes, the rest of the

Ethanol are removed, and the plate is placed off magnet. Elution Buffer (EB, 20 µl) is dispensed to each well and mixed. Purification Solution (PS, 20 µl) is added to each used well and the cleanup process is repeated once again, this time removing 40 µl of supernatant, with the final elution to 18 µl of EB. The eluate (17 µl) is added to the respective wells of the plate containing barcodes (BC, approx. 5 µl each at the time of reaction). The PCR Mix with Enzyme Mix 2 (8 µl of PE, prepared manually of PCR and E2) is added to each well in use and mixed thoroughly. The plate is moved to the thermolocator where it is held at +10 °C. The pipetting head of the robot is moved away to make the resulting plate accessible to the operator, and the robot stops. Phase 1 is finished. After this step, each used well of the plate contains cDNA solution cleaned from oligodT beads and second-strand-synthesis reaction components. The solution contains primers, enzymes, and all other required PCR components, and is ready for thermocycling.

Phase 2–PCR Amplification

The operator takes the plate out of the robot as instructed by the program, seals it with PCR foil, and places it in the thermocycler. Thermocycling is performed according to the specified program (see p.25). Preferably, Phase 2 and Phase 3 (see below) of the protocol are run in separated rooms to avoid any possible contamination of pre-PCR samples. This amplification yields a library containing complete adaptor sequences required for cluster generation.

Phase 3–PostPCR on the Zephyr NSG Workstation

For this phase, the standard PostPCR SPRI Cleanup Application for the Zephyr NGS Workstation is used. The PCR product (25 µl from each well) is purified using 25 µl of Purification Beads, washed twice with Ethanol, and then eluted in 20 µl of EB.

The samples are dispensed into the Purification Beads plate. At this stage, the user is prompted to remove the original sample plate from the deck. This is the only manual intervention required during the run in Phase 3. The samples and the Purification Beads are mixed and transferred to a clean plate. The plate is incubated for 10 minutes at 25 °C with 500 rpm agitation on a thermoshaker, then moved to the magnet, and incubated for another 10 minutes. The supernatant is removed and discarded. Separation is repeated once again.

An Ethanol (80%) wash step (150 µl per well) with 30 second incubation time is performed twice. The remaining Ethanol is removed from the sample plate, and the plate is moved to the shaker where the beads can dry for 3 minutes.

The Elution Buffer (EB, 20 µl) is transferred to the sample plate, mixed, incubated for 3 minutes, and mixed again. The sample plate is then placed to the magnet, the beads are separated for 3 minutes, and the eluate without beads is transferred to a clean plate. Phase 3–PostPCR is finished, and the user is prompted to remove the resulting plate from the deck.

After this step the amplified and purified libraries (20 µl in each well) are available in the resulting plate. The autoSENSE mRNA-Seq V2 protocol is finished.

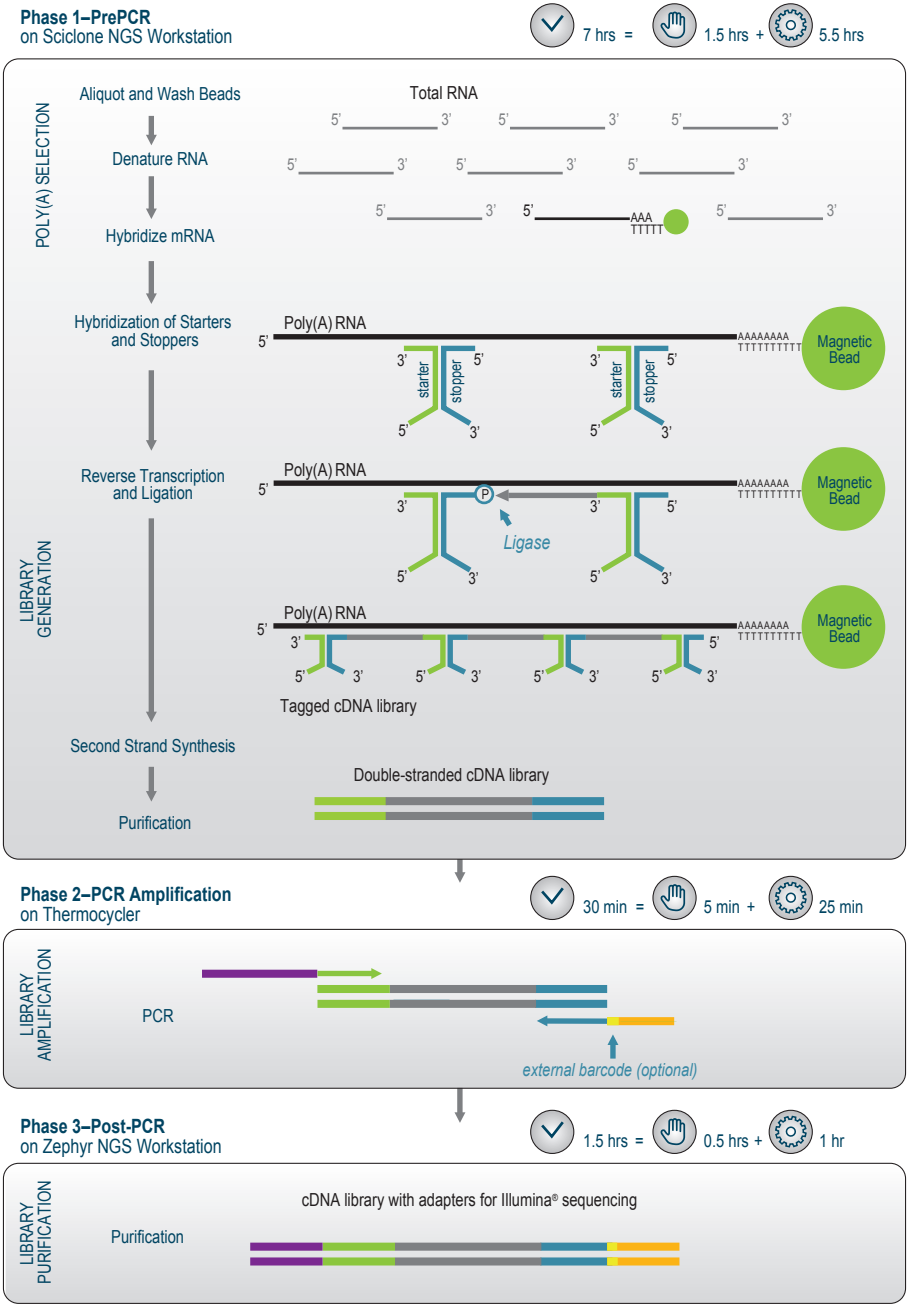


Figure 1: Overview of the autoSENSE mRNA-Seq V2 workflow.

3. Kit Components and Storage Conditions

For an autoSENSE mRNA-Seq V2 run, you will need the SENSE mRNA-Seq Library Prep Kit V2. Optionally, you may need additional reagents like **BD** ● and **OIL** ❍ , which are included in the Automation Module for SENSE mRNA-Seq V2..

A. SENSE mRNA-Seq Library Prep Kit V2

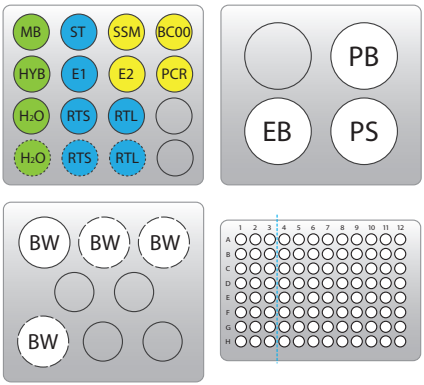


Figure 2. Location of kit contents in a 96 prep SENSE mRNA-Seq Library Prep Kit V2. For the 24 prep kit the purification solutions (PB, PS, EB) and BW are provided in smaller containers and the dotted solutions of the reagent box (top left corner) are missing. External barcodes are provided in a 96-well plate (bottom right corner). For 24 prep kit, columns 1-3 (up to blue dotted line) and for 96 prep kits all columns are filled with barcodes, respectively. For BC sequences see p.36.

Kit Component	Tube Label	Volume provided		Storage
		24 preps	96 preps	
Magnosphere MS150 / OligodT Beads	MB ●	300 µl	1200 µl	-20 °C
RNA Hybridization Buffer	HYB ●	300 µl	1200 µl	-20 °C
Molecular Biology Grade Water	H_2O ●	1500 µl	3000 µl	-20 °C
Reverse Transcription and Ligation Mix Short	RTS ●	500 µl	2000 µl	-20 °C
Reverse Transcription and Ligation Mix Long	RTL ●	500 µl	2000 µl	-20 °C
Starter/Stopper Mix	ST ●	60 µl	240 µl	-20 °C
Enzyme Mix 1	E1 ●	90 µl	360 µl	-20 °C
Second Strand Synthesis Mix	SSM ●	270 µl	1080 µl	-20 °C
Enzyme Mix 2	E2 ●	70 µl	250 µl	-20 °C
PCR Mix	PCR ●	280 µl	910 µl	-20 °C
BC00	BC00 ●	44 µl	44 µl	-20 °C
Barcode Plate (96-well plate)	BC	Set 1-3	Set 1-12	-20 °C
Purification Beads	PB ○	1320 µl	5280 µl	+4 °C
Purification Solution	PS ○	3660 µl	14640 µl	+4 °C
Elution Buffer	EB ○	3750 µl	15000 µl	-20 °C/+4 °C
Bead Wash Buffer	BW ●	24000 µl	96000 µl	+4 °C

Upon receiving the SENSE kit, store **PB** ❍ and **PS** ❍ at +4 °C. The rest of the kit should be stored in a -20 °C freezer. **EB** ❍ can be stored either at +4 °C or -20 °C. Before use, check the contents of **PS** ❍ and **BW** ● which may precipitate during shipping. If a precipitate is visible or the content appears milky, incubate at 37 °C until buffer components dissolve completely.

B. Automation Module for SENSE mRNA-Seq V2

Figure 3. Location of kit contents in the Automation Module for mRNA-Seq V2 kit.

Kit Component	Tube Label	Volume provided	Storage
		96 preps	
Bead Diluent	**BD** ●	1000 µl	+4 °C
Silicone Oil	**OIL** ❍	3000 µl	+4 °C

Upon receiving the Automation Module kit, store **BD** ● and **OIL** ❍ at +4 °C.

4. User-supplied Consumables and Equipment

Reagents

Reagent	Supplier
80% Ethanol (about 106 ml for 96 reactions)	Various

General Laboratory Equipment and Supplies

Equipment	Supplier
Suitable pipette tips (tips with aerosol barriers recommended)	Various
Thermocycler (Bio-Rad HSP-96 PCR-plate compatible)	Various
Microfuge	Various
Vortexer	Various
Microplate centrifuge	Various
Plate seals	Various
Magnetic rack (for manual bead wash)	Various

Sciclone NGS / Zephyr NGS Workstation Accessories

Accessory	PerkinElmer Part No.
Agencourt® 96-ring magnet (1 for Sciclone, 1 for Zephyr)	CLS 128316
Magnet spacer (1 for Sciclone)	CLS 133514
Inheco 96-well adapters (2 for Sciclone, 1 for Zephyr)	CLS 128372
Inheco 96-well adapter/shaker (1 for Sciclone, 1 for Zephyr)	CLS 100852
Inheco 384-well adapter (1 for Sciclone)	CLS 100853

Optional Equipment and Material (for RNA Quantification and Quality Control)

Equipment and Material	Supplier
UV-spectrophotometer	Various
Automated microfluidic electrophoresis station	Agilent Technologies 2100 Bioanalyzer, PerkinElmer LabChip GX II
qPCR machine and library standards	Various
Benchtop fluorometer and appropriate assays	Various
Agarose gels, dyes, and electrophoresis rig	Various

The complete set of material and labware necessary for RNA extraction and quality control is not listed. Consult Appendix B (p.29) for more information on RNA quality. Consult Appendix D (p.34) for information on library quantification methods.

Sciclone NGS / Zephyr NGS Software Requirements

Maestro software 6.0 patch 44 or newer must be installed on the control PCs for both Sciclone NGS and Zephyr NGS.

On-deck Consumables

Consumable	Description	PerkinElmer Part No.	Original Vendor and Part No.	No. Used per Run (96 samples)
PCR Plates	Bio-Rad 96-well Full skirt Hard-shell plate	600 8870	Bio-Rad HSP-9631	12
384-well plate	Microplate 384-well, round bottom	600 8890	Corning, Inc. 3672	1
Boxed Tips	Pipette Tip 150µl , filtered, sterile	111426	-	11
Deepwell Plates	Deepwell-06 POS, Square 2.0 mL well, Polypro, Seahorse	600 8880	Seahorse Bioscience 201379-100	5
Lids	946 Lid-Universal, Robotic Friendly, Polystyrene	600 0030	Seahorse Bioscience 200856-100	3

5. General Guidelines

Liquid Handler Usage

- Always wear gloves when touching the liquid handler casing, the deck, the Inheco controllers, and any other parts of the robotic workstation.
- Always wear gloves when touching any consumables on deck.
- When placing the consumables on the deck, make sure they are securely positioned.
- Before each run, lubricate the O-Rings of the Disposable Tip Array with the lubricator box. See Sciclone User manual or Zephyr User manual.
- At some points of the protocol used consumables such as lids or plates are discarded via the waste chute. Make sure a suitable basket or a box can hold the consumables falling out of the chute and prevent any potential damage to the surrounding equipment.
- Do not use consumable types other than specified in this document, even if they seem to

be very similar to the specification. The liquid handler is programmed to the dimensions and specifications of the consumables mentioned and even minor physical differences may cause malfunction of the handler. Ask Lexogen if in doubt.

RNA Handling During Run Preparation

- RNases are ubiquitous and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e. RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) according to the manufacturer's instructions.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Manual Bead Handling

- Prior to transferring the oligodT beads to the plates, the beads must be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or bottom of the tube.
- Beads may stick to certain pipette tips, in which case removing the beads from the inside of the tip may be impossible. Avoid resuspending by repeated pipetting and instead resuspend by vortexing if this occurs with your tips.
- The following guidelines mainly apply to the manual washing step:
  - Beads are superparamagnetic and are collected by placing the tube in a magnetic stand. The time required for complete separation will vary depending on the strength of your magnets, tube thickness, viscosity of the solution, and the proximity of the tube to the magnet. Separation time may need to be adjusted accordingly. When fully separated, the supernatant should be completely clear and the beads collected at one point or line on the wall of the tube.
  - To remove the supernatant the tube containing the beads has to stay in close contact with the magnet. Do not remove the tube from the magnetic stand when removing the

supernatant, as the absence of the magnet will cause the beads to become resuspended again.

- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant but before adding the next reagent. Beads can be resuspended by vortexing but make sure that beads are not deposited on the tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the tube briefly with a benchtop centrifuge.

Pipetting and Handling of (Viscous) Solutions

- Enzyme mixes, RTS, RTL, and PS are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- When drawing up liquid the tip should be dipped 3 to 5 mm below the surface of the liquid, always at a 90 degree angle. Do not dip the tip in any further as viscous solutions tend to stick to the outside of the pipette tip.
- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle, and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile whereas the pipette itself is not. If necessary tilt the bottle to bring the liquid closer to the opening and facilitate pipetting.

Miscellaneous

- Ensure that adequate volumes of all reagents and the necessary equipment is available and set to the proper temperatures before beginning the protocol.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly and centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep enzyme mixes at -20 °C until right before use or store in a -20 °C benchtop cooler.

- The step requiring a thermocycler has been tested with a maximum ramp speed of 5 °C/sec before denaturation and extension and 2.5 °C/sec during primer annealing. While these ramp speeds are typical for most modern thermocyclers, some models can exceed these rates, and ramp speed may need to be decreased to ensure efficient annealing.
- To keep track of the pipetting steps, tick the respective cells in the worksheet indicating a completed column of a plate.
- Inspect each plate after you have finished pipetting. If bubbles are present in the liquids, spin the plate shortly. Protect the content of the plate with a lid when spinning. For plate P7, we recommend to spin the plate after transferring all reagents but the magnetic beads (column 12). Transfer the magnetic beads after spinning to keep the beads suspended.
- Label each plate immediately after preparation using a lab marker. To enable easy identification on the deck, we recommend to mark the plate in the upper left corner on the top surface.

6. Software Download and Installation

NOTE: Installing the application on the instrument for the first time is similar to other protocols on the PerkinElmer liquid handlers but requires some experience and skills. Therefore, we recommend asking for Lexogen support. The following description is just for your information.

Download

All software components of the autoSENSE mRNA-Seq V2 kit can be downloaded from Lexogen website at www.lexogen.com. Please download the latest available release and always read the respective Releases Notes file which may supersede the instructions given in this manual. Unpack the archive and copy the files to the following destination folders on your PC connected to the Sciclone:

Folder LEXOGEN with all subfolders to
C:\ProgramData\CaliperLS\Maestro

File LEX_autoSENSE_V2_prePCR_YYYY-MM-DDn.zip to
C:\ProgramData\CaliperLS\Maestro\Exported Archives

If you are updating an existing installation, we recommend to rename the old files and folders first, then load the new versions. You can keep the old files for reference.

First Start

Start the Maestro software, open a new application (**File > New Application**) and import the archive file you have just downloaded (**File > Import > Import Archive**). You find the file in C:\ProgramData\CaliperLS\Maestro\Exported Archives. In the **Import Archive Options** dialog, keep the **Current Application** and **VSTA Macros** checkboxes checked and click **OK**. You will be immediately prompted to save the application in the **Save Maestro Application** file dialog. Save the application under the suggested name.

Fine Tuning Application

The application has all critical pipetting heights identified and appropriate variables created to control those pipetting heights. The actual values of these variables used to position tips precisely at the bottom of consumable wells are instrument and application specific. Setup (teaching) must be done on the instrument which will be used for real runs.

The variables to be taught are summarized in the UTIL_SetGlobalOffsets method of the application. They are named *g_Offset_NNN* and *L_correction_N_column*. The teaching itself consists of manual control of the gantry to drive the head down until the tips touch the bottom of the wells in the respective consumable dedicated for the given location on deck. Each variable has a description with specification of its teaching conditions (location on the deck and type of consumable used at this location). The optimum values found during the manual teaching are then saved to the code of the UTIL_SetGlobalOffsets method.

When updating the application to a new version, the currently validated values of the *g_Offset_NNN* and *L_correction_N_column* have to be copied to the new application.

Dry and Wet Tests

After installing the application, we recommend to run a dry test and a wet test first. The dry test will ensure that gripper transport of plates, tips, and lids is robust, and there are no collisions with accessories and consumables. We then recommend to run the wet test using dummy reagents with fluidic behaviour close to the real pendants (ask Lexogen for a kit with dummy reagents). The wet test will ensure the liquid handling is clean and the beads are not lost during cleanups.

7. Detailed Protocol

Read all the instructions carefully prior to beginning the run. This description assumes the Application Phase 1 is already installed on the control PC of the Sciclone NGS, and the Application Phase 3 is already installed on the control PC of the Zephyr NGS.

7.1 First Preparation Steps

- Purification Beads (PB), Purification Solution (PS), and Bead Diluent (BD) need to be equilibrated to room temperature for about 30 minutes before use. They may be taken out of +4 °C storage before beginning.
- If necessary, boot up the system by first starting the Sciclone and the Inheco units, then starting the Sciclone control PC. Also start the Zephyr and its Inheco units, then start the Zephyr control PC.

7.2 Workbook Preparation

Open the Microsoft Excel workbook in this filepath of the Sciclone control PC:
C:\ProgramData\CaliperLS\Maestro\LEXOGEN\Workbooks\
autoSENSEV2 Library Prep Workbook.xls

In the worksheets *Reagent Plates PrePCR(Sciclone)* and *Reagent Plates PostPCR(Zephyr)* of this workbook (Figure 4), you will find pipetting schemes as well as reagent preparation guidelines for the respective protocol phases. Fill the yellow marked cells in the header of the *Reagent Plates PrePCR(Sciclone)* worksheet (assay ID, date, number of samples, the bead prewashing option, etc.). All the pipetting schemes and reagent preparation recipes will be automatically adapted following your input. Do not modify any other content of the worksheet manually, except for the yellow-marked cells.

The **Assay ID** and **Date** entries do not affect the run in any way and are for reference only.

For the **Number of samples**, you can select any multiple of 8, from 8 to 96 (the application processes full columns of 8 samples each). The standard setting is 96 samples. If you intend to process less samples (e.g., for testing), refer to the following note.

Processing less than 96 Samples

The application which is setup to use the first N sample columns only, will always take the barcodes from the columns 1, 2, … , N of the barcode plate. In the kit barcode plate, however, the first columns might have been used in the previous run. Therefore, the barcodes have to be manually transferred from the kit barcode plate to the wells in the first N columns of the bar-

code plate placed on the deck prior to application start. See Chapter 7.3 Plate preparation / P6 Plate – Barcodes.

Manual Beads Prewashing

In the **Beads in P7 prewashed?** cell, select between **YES** (operator has prewashed the beads manually), or **NO** (the beads have been pipetted into the P7 plate directly from the kit stock). Note that this only applies to the oligodT beads included in the kit (not to the Purification Beads).

Beads in P7 prewashed (option YES)
This option is recommended for high-throughput processing, as a larger amount of the oligodT beads (for several machine runs) can be prewashed in a batch. By selecting this option, the machine run time can be reduced. Manual bead wash will, however, increase the manual preparation time. Consumption of tips or other consumables is not affected. The manual bead washing guide can be found in Appendix A (p.28).

Beads in P7 raw (option NO)
Use this option to enable fully automated bead washing. You can use this option for processing smaller amounts of samples.

For comparison, the protocol parameters for processing 96 samples (1 full plate) are summarized in the following table:

96 samples, Phase 1–PrePCR only	Beads in P7 prewashed (option YES)	Beads in P7 raw (option NO)
Manual preparation time [minutes]	90	80
Machine run time [minutes]	315	330
Tip consumption [number of tip boxes]	7	7

Save the modified Microsoft Excel workbook under the original name in the original filepath. It is recommended to print out the respective worksheets at this stage, and use the printout as your reagent preparation guide.

Low RNA Input Option

In the **RNA input below 50 ng:** cell, select between **YES** or **NO**. The only difference in the protocol is the incubation time in the hybridization step (20 min if **YES**; else 5 min).

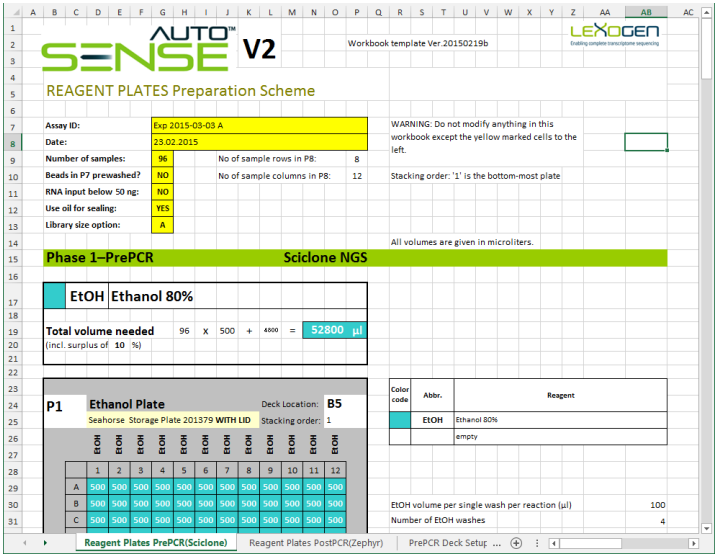


Figure 4: A fragment of the autoSENSE V2 Workbook. Note that the actual layout of the spreadsheet in the software distribution may differ slightly from the layout shown.

Oil Sealing Option

In the **Use oil for sealing:** cell, select between **YES** or **NO**. If oil is used for sealing the reaction wells during heating (option **YES**), the Phase 1-PrePCR of the protocol does not require any operator intervention. Note that oil is included in the Automation Module for SENSE for mRNA Seq V2. Alternatively, manual film sealing can be used. In this case (option **NO**), the liquid handler stops and the operator is asked to take the plate, seal it with film, put it back on deck, and resume run.

Library Size Option

In the **Library size option:** cell, select one of the **A** to **C** options. The recipes of the reagents will be adapted according to the required library size. Refer to Appendix C (p.32) for detailed instructions on this selection.

7.3 Plate Preparation

For reference, the plates used in the protocol are named P1, P2, etc. The plates P1 to P5, P7, and P8 have to be filled with reagents by the operator. For processing 96 samples, the plate P6 is the 96 prep kit Barcode Plate . For smaller number of samples, the P6 plate may have to be prepared by the operator. The numbering of the plates corresponds to the recommended preparation sequence. For the deck setup, you will also need some empty plates. Some of them will be used

for reagent transfer during the protocol, and must be clean and sterile. Some other plates are used as spacers, can be non-sterile and may be reused.

Understanding the Plate Preparation Worksheets

The detailed plate setup is described in the Microsoft Excel workbook. The worksheets *Reagent Plates PrePCR(Sciclone)* and *Reagent Plates PostPCR(Zephyr)* also contain recipes for master mixes used in the run.

Refer to Figure 5 for the explanation of the worksheets. We recommend to follow the worksheet from top to bottom and to prepare the plates one after another in the given order. For each plate, check whether the reagents to be filled in this plate are readily available in the kit, or a master mix (or more master mixes) has/have to be prepared first. Prepare all master mixes first, then proceed with transferring the reagents into the plate wells, column after column. Cover the plate with a lid if this is indicated.

Specific instructions for selected individual plates are given below.

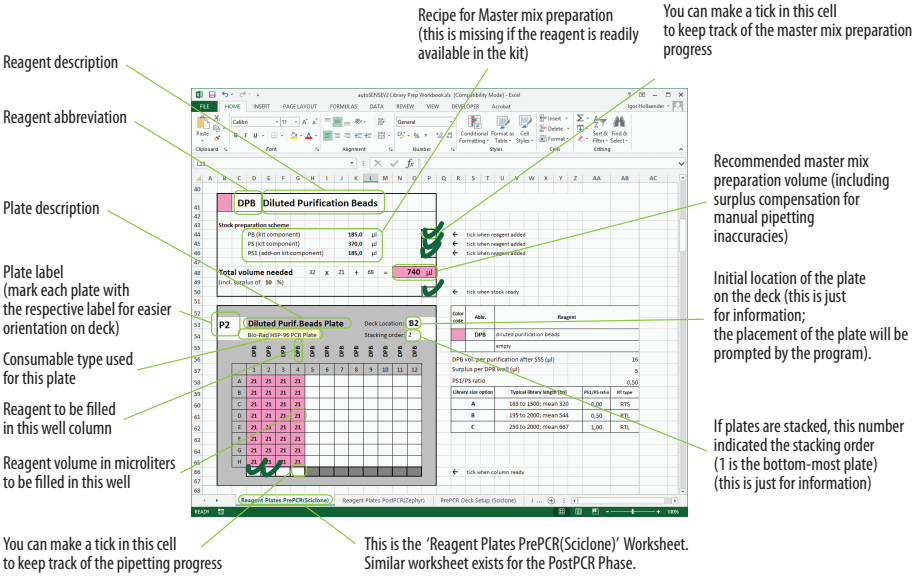


Figure 5: Understanding Worksheet.

P6 Plate – Barcodes

The kit Barcode Plate comes prefilled with the barcoded primers and sealed with cap stripes. The plate can be either used on the deck (for a full run using all barcodes, i.e., 24 barcodes from the 24 prep kit, or 96 barcodes from the 96 prep kit), or the selected barcodes can be transferred to a new plate.

- The kit barcode plate has to be stored at -20 °C.
- Before the run, thaw the plate for 5 minutes in a thermocycler at 25 °C. Visually inspect the wells to ensure that they all are completely thawed.
- Centrifuge the plate shortly to collect all of the barcode at the bottom of the well.
- Remove the lid, remove *all* cap stripes, and cover the plate with the lid again. Make sure the lid and the plate are free from any rests of the sticky tape, in particular at the side walls. The plate is now ready to be placed on deck as the P6 plate.

If **processing less than 96 (24) samples** (i.e., you do not intend to use up all barcode primers from the kit Barcode Plate in this run), proceed as follows:

- Using a pipette or an 8-tip multichannel pipettor, transfer the full volume of the barcodes in the selected wells of the kit Barcode Plate to a new Bio-Rad HSP-96 plate (the new P6 plate). Fill the new P6 plate column-by-column, starting from column 1. Cover the new P6 plate with a new lid after filling. The new P6 plate is now ready to be placed on deck.
- We recommend to recap the used empty wells of the kit Barcode Plate to avoid contamination at the next use.
- Put the plastic cover that came with the kit Barcode Plate back on the plate.
- Refreeze the kit Barcode Plate and store at -20 °C.
- It is recommended to use the kit Barcode Plate for a maximum of four thaw/refreeze cycles.
- The filling scheme of the kit Barcode Plate with barcode sequences can be found on p.36.

P7 Plate – Reagents

Thaw the SENSE reagents stored at -20 °C. Ensure that each supplied reagent has been fully thawed, gently mixed, and spun down before use. After adding the appropriate volumes of reagents in nuclease-free tubes, gently mix and spin down again to collect all liquid at the bottoms of the tubes.

Keep the master mixes on ice and promptly return unused portions of reagents to -20 °C storage.

Depending on your selection, the worksheet will guide you to fill in the raw (MBR) or the prewashed (MBW) beads. The manual bead washing guide is in Appendix A (p.28).

Aliquot all reagents except the oligodT beads into the plate. Pipet carefully into the bottom of the wells and avoid trapping air or creating bubbles. If necessary, spin the plate down briefly in

a plate centrifuge to ensure all reagents are at the bottom of the wells. After this optional spinning, finish the plate preparation by transferring the oligodT beads (beads should not be spun to keep them suspended).

Store the plate on ice or at +4 °C until you place it on deck.

P8 Plate (Samples)

Prepare this plate as the last one in the preparation process, immediately before starting the run. Observe the general recommendations for RNA sample handling given on p.14.

Cover the plate with a lid after filling and store on ice. Place the plate on the deck when prompted by the application. This will ensure the thermolocator at the respective position is already pre-cooled to the plate storage temperature.

7.4 Starting the Application on Sciclone NGS

Start the Maestro software on the Sciclone control PC. Load the autoSENSE V2 application by selecting **File > Open Application**. In the **Open Maestro Application** dialog, select the LEX_autoSENSE_V2_prePCR application (make sure you select the correct one if you have stored more versions) and click **Open**. If the application is loaded properly, a window with the LEX_SENSE_00_Main method will be shown at the top (Figure 6).You do not need to setup the deck prior to starting the application. Instead, wait for the application to prompt you to do so.

Start the application main method. An introductory application window will appear. Click the green **Continue** button. Throughout the application, green controls indicate the suggested (or typical) way of proceeding. The application window will always show the actual processing phase.

After a while, the application will show the parameters from the workbook. Check that the values in the workbook have been read correctly (for a quick check, make sure the Assay ID shown corresponds to your entry in the workbook). The most frequent cause of a problem (the application stops with an error message) is that the workbook is not at its correct location or is not readable. If the workbook has been read correctly, press **Continue**.

7.5 Sciclone NGS Deck Setup

The application will now show a **Deck Setup Visual Check Dialog**. Browse through all pictures and hints presented. At this stage, you can place the consumables and the prefilled plates to their respective positions on deck as instructed by the pictures. The thermolocators holding the plates with sensitive reagents are now at the required storage temperature and the head of the liquid handler has moved to the back to free the manipulation space for the operator. Make sure your deck has been set up according to the instructions.

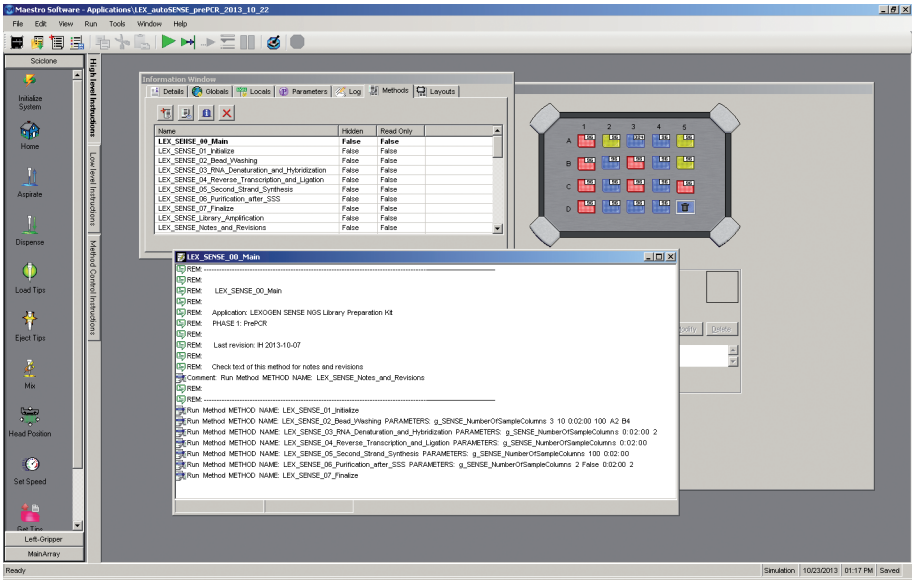


Figure 6: The Maestro window with the autoSENSE V2 application loaded.

7.6 Phase 1–PrePCR Run

Pressing **Start run** in the Deck Setup Visual Check dialog will start the liquid and material handling process of the protocol. If Oil is used for sealing, no operator intervention will be required until Phase 1–PrePCR is over, which will take about 5.5 hours. If film is used for sealing, the machine will stop after about 3 hours of operation and ask operator for sealing the plate, then runs the thermal cycle, and an intervention for unsealing the plate is required again. For the detailed step-by-step guide to the protocol, see the Application Workbook, worksheet *Detailed Protocol*.

7.7 After Phase 1–PrePCR Completion

When Phase 1–PrePCR is complete, the application will stop. Close the final dialog. The plate with the reagents prepared for the PCR amplification is located at the thermolocator T3 (location D2) and held at +10 °C.

Remove used tip boxes from the deck. Discard used plates. Empty the tip waste.

7.8 Phase 2–PCR Amplification

Take the plate from location D2, seal it with a film. If bubbles are present in the liquid, spin the plate down shortly. Place the plate on a thermocycler. The recommended library amplification program is

- 98 °C, 30 sec
- Repeat **13** times
  - 98 °C, 10 sec
  - 65 °C, 20 sec
  - 72 °C, 30 sec
- 72 °C, 2 min
- 10 °C, ∞

Set the thermocycler for 30 µl of PCR Mix. Use a heated lid (recommended temperature 105 °C) to prevent condensation. See Appendix B: RNA Requirements – PCR Cycles (p. 29) for information regarding optimization of library amplification. Start thermocycling.
We recommend to use the time for preparing the run on the Zephyr NGS (see next chapter 7.9).

7.9 Zephyr NGS Deck Setup

The Post-PCR SPRI cleanup is a separate Maestro application, running on the Zephyr NGS workstation (a different liquid handler for postPCR sample processing to avoid any possible cross-contamination of the pre-amplification samples).

Note: Post-PCR SPRI cleanup applications are available for both Sciclone NGS and the Zephyr NGS workstations. The instructions here are for running the autoSENSE V2 post-PCR SPRI application on the Zephyr NGS workstation. Ask Lexogen for a different setup.

Fill the plates for Phase 3–PostPCR following the guidelines in the respective worksheet of the Microsoft Excel workbook. Make sure the Purification Beads are at room temperature and mixed well prior to pipetting. Place the plates on deck according to the workbook.

7.10 Phase 3–PostPCR Run

Start the application main method. In the introductory dialog, fill the entries like follows (Figure 7): **Number of columns** according to the actual setup; **Sample Volume** 25 µl; **Bead Volume** 25 µl; **Elution Volume** 20 µl.

You will be prompted to check the deck setup, and to start the liquid and material handling process. Shortly after start, you will be prompted to remove the original sample plate from the deck. This will be the last manual intervention required. The whole process will take about 55 minutes for a plate of 96 samples.

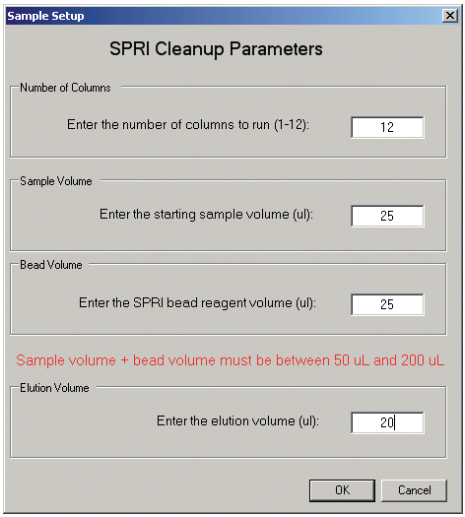


Figure 7: Introductory dialog of the Phase 3-PostPCR.

7.11 After Phase 3-PostPCR Completion

When the Phase 3–PostPCR is complete, the application will stop. The plate with the amplified and purified library is located at C2.

Remove used tip boxes from the deck. Discard used plates. Empty the tip waste.

The autoSENSE mRNA-Seq V2 application is completed.

8. Appendix A: Manual oligodT Bead Washing – Detailed Guide

Manual bead washing saves liquid handler machine time. It is a relatively easy protocol which can be done in a larger batch so a stock of washed oligodT beads (MBW) is then prepared for usage in several machine runs.

Equipment: magnetic rack, 1.5 ml tubes.

Reagents required:
In the Application Workbook / worksheet *Reagent Plates PrePCR(Sciclone)*, select the **YES** option for the **Beads in P7 prewashed?** selector, fill the required **Number of samples**, then check the amount of washed oligodT beads (MBW) required for a single run in the *Consumption Summary* worksheet. Calculate the batch volume of washed beads you want to prepare.

100 µl beads can be conveniently washed in a 1.5 ml tube. 12 preparations (100 µl each) are required for a 96 library prep run.
To prepare 100 µl of washed beads (MBW) you need:

100 µl	of raw oligodT Beads (MBR, kit component),
2100 µl	of Bead Wash Buffer (BW, kit component).

Protocol (to prepare 100 µl of washed beads):

1	Mix the oligodT beads (MBR) thoroughly.
2	Transfer 100 µl of the resuspended raw beads (MBR) into a new 1.5 ml tube.
3	Place the tube in a magnetic rack and let the beads collect for 2 minutes. Remove and discard the supernatant.
4	Remove the tube from the magnetic rack and add 1000 µl Bead Wash Buffer (BW). Re-suspend the beads and transfer the tube to the magnetic rack. Let the beads collect for 1 minute, then remove and discard the supernatant.
5	Repeat the washing step once again (for a total of two washes).
6	Resuspend the beads in 100 µl Bead Wash Buffer (BW). The resulting suspension is the MBW reagent.
7	Store the MBW stock at +4 °C.

9. Appendix B: RNA Requirements - PCR Cycles

RNA Amount

High quality mRNA-Seq data relies on high quality input RNA. The amount of total RNA required for autoSENSE V2 depends on the poly(A) RNA content of the sample in question. This protocol was tested extensively with various mouse tissues and human reference RNA. Typical inputs of 500 ng total RNA for mRNA-rich tissues (such as kidney, liver, and brain) or 2 µg total RNA for tissues with lower mRNA content (such as lung and heart) generate high quality libraries for paired-end 50 nt sequencing (PE50) with 13 cycles of library amplification.

RNA inputs down to 10 ng total RNA from mRNA-rich samples such as Universal Human Reference RNA (UHRR) were used for successful library generation with SENSE mRNA-Seq V2. However, reducing the input RNA also requires increasing the number of PCR cycles during the PCR amplification step. **ATTENTION:** If using 50 ng total RNA input or less, set the **RNA input below 50 ng:** cell to **YES**. For reduced RNA inputs we also strongly recommend performing a qPCR assay (see below) to determine the appropriate cycle number for the endpoint PCR.

The input requirements for your particular experiment may be different. If RNA input is not sufficient, either due to naturally low poly(A) RNA content or degraded RNA, additional cycles of library amplification may be necessary. However, as additional cycles of library amplification may increase the proportion of PCR duplicates, it is more desirable to increase the amount of input RNA (if possible for your application) rather than to rely on extra PCR cycles to increase library yield.

qPCR to Determine the Exact Cycle Number of Your Endpoint PCRs

For determining the cycle number of your endpoint PCR we recommend to make a run with (as an example) 4 reactions (in the workbook, set the protocol for 8 samples, but only fill four of the wells, e.g., A1 to D1 with reagents), each with 500 ng or 2 µg of total RNA according to the expected poly(A) content. Use a new barcode plate filling 8 µl of Barcode 00 (**BC00** ●) to the used wells of the first column of the P6 plate. Run the Phase1-PrePCR phase of the protocol. Then add SYBR Green I (or an equivalent fluorophore) to the PCR reaction to a final concentration of 0.1x. For 0.1x SYBR Green I add 1.2 µl of a 1:4,000 SYBR Green I dilution (diluted in DMSO) to each reaction. The total PCR reaction volume will be 31.2 µl. SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually. Overcycle this initial qPCR (30 cycles or even more if little input material was used) and then determine the fluorescence value at which the fluorescence reaches a plateau. Calculate where the fluorescence is 25 % of the maximum, and this is the cycle number you should use for the endpoint PCR. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.

RNA Integrity

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA 6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN), and we recommend a RIN score of 8 or greater for optimal sequencing results. Typically, such samples have easily detectable rRNA peaks and a comparatively low abundance of short RNAs, which can arise from both intact short transcripts as well as from RNA degradation. Libraries can also be generated from lower quality RNA, but this may lead to 3'-bias in sequencing results.

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should also be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260/A230 ratio. Phenol also has an absorption maximum between 250 and 280 nm which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of gDNA, which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids much more intensely than double-stranded, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel, gDNA can appear as either a dark mass which remains in the slot if relatively intact or as a high molecular weight smear if it has been sheared during extraction. autoSENSE V2 libraries generated from samples containing gDNA may have an increased number of intergenic reads or lower strandedness.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA, such as the SPLIT RNA Extraction Kit (Cat. No. 008.48) by Lexogen. However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. We do not recommend DNase treatment as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided and the enzyme deactivated by other means such as phenol/chloroform extraction or silica column purification.

RNA Storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater (Life Technologies Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Avoid frequent freeze/thaw cycles as RNA might be sheared.

ERCC RNA Spike-in Controls

To enable the hypothesis-neutral calculation of strandedness, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the ERCC RNA spike-in controls (Ambion Inc.). These sets of RNAs have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligning to the genome.

10. Appendix C: Adjusting Library Size

The size of autoSENSE mRNA-Seq V2 libraries can be adjusted to the desired sequencing length. This is accomplished by modulating the insert range of the library generated during RT/ligation and by using different size selections during purification.

The SENSE mRNA-Seq kit is offered with two different Reverse Transcription and Ligation Mixes. As shown in the table below, RTS will produce libraries with shorter mean insert sizes, while RTL generates libraries with longer inserts. Additionally, the desired library size is further fine-tuned by modulating the magnetic bead-based purification (mix of PB, PS, and BD).

For convenience, you can readily select between three typical library size settings. the selection is made in the **Library size option:** cell of the Workbook. Please refer to the table below to see which size selection is appropriate for your desired read length.

Depending on your selected insert range the number of PCR cycles during library amplification varies slightly. **ATTENTION:** All reference values shown here refer to 500 ng total RNA starting material (Universal Human Reference RNA, UHRR). If using less input RNA or RNA with low mRNA content, further cycles need to be added. In this case we strongly recommend performing the qPCR assay as described in Appendix B, p.29.

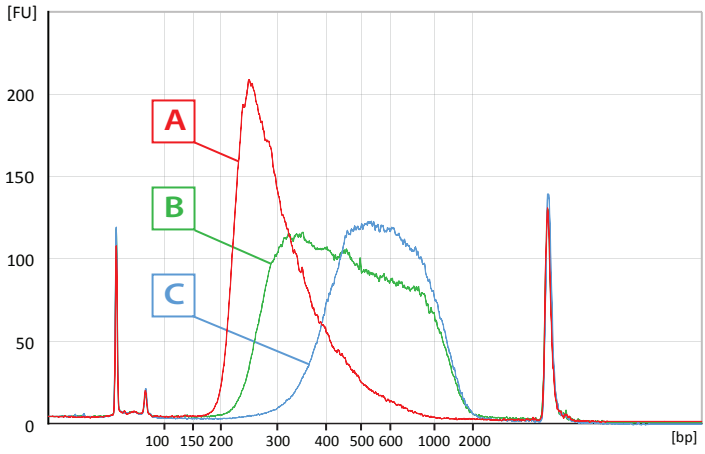


Figure 8: Bioanalyzer traces of typical libraries representing size options A,B, and C.

Sequencing length up to	Library Size Option in the Workbook	Library*			Insert**				Library Yield***	
		Start [bp]	End [bp]	Mean size*	Mean size	>100 nt	> 200 nt	> 300 nt	ng/µl	nM
≤PE50	A	165	1500	320	208	82 %	29 %	9 %	3.29	17.3
PE100	B	195	2000	544	422	96 %	74 %	47 %	3.01	11.2
≥PE150	C	250	2000	667	545	96 %	94 %	80 %	1.87	5.3

* For multiplexed (externally barcoded) libraries. Libraries prepared without external barcodes (BC00) are 6 bp shorter.
** Mean insert size is the library size minus 122 bp (116 bp adaptor sequences + 6 bp barcode).
*** 500ng total RNA input, 13 PCR cycles
PE: Paired-End Sequencing

The combinations (RTS/RTL/PB/PS/BD) recommended for the different sequencing lengths in the table above were selected to provide a good balance between maximizing the total number of bases sequenced and an even coverage distribution. These settings are optimal for gene expression (counting) applications. If full-length transcript assembly or isoform detection (e.g., splice variants) is important, we recommend using buffer combinations for the next shorter library size. This should provide insert sizes slightly below the chosen read length of the run. While losing some sequencing space by reading into the 3' adapter sequence the coverage along the transcripts will improve.

11. Appendix D: Library Quality Control

Quality control of finished autoSENSE V2 libraries is highly recommended and can be carried out using various methods depending on available equipment. A thorough quality control procedure should include the analysis of both the concentration and the size distribution of libraries.

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become the de facto standard for many NGS laboratories and generates information regarding library concentration and size distribution. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Agilent Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies Inc.). For high throughput applications instruments such as LabChip GX II (PerkinElmer) or 2200 TapeStation (Agilent Technologies Inc.) are recommended. Typically, 1 µl of the autoSENSE V2 library is sufficient for analysis. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl for TapeStation and 10 µl for LabChip GX II) .

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished autoSENSE V2 library is calculated by comparing Cq values to a set of known standards. While generating more accurate quantification, these assays do not supply the user with information regarding library size distribution and/or linker-linker content. The use of such an assay for quantification in combination with Bioanalyzer analysis for size distribution is highly recommended.

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

Typical Results

Typical concentrations are between 5.3 – 17.3 nM (1.9 – 3.3 ng/µl), which are well suited for cluster generation without further processing. A shorter side-product caused by the direct ligation of starter/stopper heterodimers to one another is sometimes visible at ~135 bp and should compose no more than 0 – 3 % of the total library. Higher proportions of this side-product can indicate problems during library preparation.

A second peak in high molecular weight regions (between 1000–9000 bp) is an indication of overcycling. This might have an impact on library quantification and PCR duplication rate. Performing the qPCR reaction to determine the cycle number of your endpoint PCR as recommended on page 29 should prevent overcycling. Still, even overcycled PCRs can be used for subsequent sequencing reactions without significantly compromising your results. However, for further experiments using the same input RNA please adjust your cycle number accordingly or take advantage of the qPCR option.

12. Appendix E: Multiplexing

autoSENSE mRNA-Seq V2 libraries are multiplexed. Barcodes are introduced as standard external barcodes during the PCR amplification step. The barcodes are 6 nt long. The kit barcode plate comes prefilled with barcodes according to the following table.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC01: ACATTA	BC02: GGTGAG	BC03: CGAAGG	BC04: AAGACA	BC05: TAATCG	BC06: CGCAAC	BC07: AATAGC	BC08: TTAACT	BC09: AATGAA	BC10: GATTGT	BC11: ATAAGA	BC12: GCCACA
B	BC13: GAACCT	BC14: CGGTTA	BC15: AACGCC	BC16: CAGATG	BC17: GATCAC	BC18: CGCGGA	BC19: CCTAAG	BC20: GGCTGC	BC21: ACCAGT	BC22: GTGCCA	BC23: AGATAG	BC24: TCGAGG
C	BC25: ACAACG	BC26: GCGCTG	BC27: CAAGCA	BC28: GTTACC	BC29: CTCTCG	BC30: CCAATT	BC31: TTCGAG	BC32: CGTCGC	BC33: TGTGCA	BC34: ACCGTG	BC35: ATACTG	BC36: ATGAAC
D	BC37: AGTTGA	BC38: GACGAT	BC39: CACACT	BC40: CAGCGT	BC41: TGCTAT	BC42: TCTTAA	BC43: CCGCAA	BC44: CTCCAT	BC45: GTCAGG	BC46: ACGTCT	BC47: GAGTCC	BC48: GACATC
E	BC49: AGGCAT	BC50: ACCTAC	BC51: TGGATT	BC52: GCAGCC	BC53: CGCCTG	BC54: CCGACC	BC55: TATGTC	BC56: TGACAC	BC57: ACAGAT	BC58: AGACCA	BC59: GCTCGA	BC60: ATGGCG
F	BC61: GAAGTG	BC62: AGAATC	BC63: GCGAAT	BC64: CGATCT	BC65: CATCTA	BC66: AAGTGG	BC67: TGCACG	BC68: TCGTTC	BC69: ACACGC	BC70: GTAGAA	BC71: AGTACT	BC72: GCATGG
G	BC73: AACAAG	BC74: AACCGA	BC75: TGGCGA	BC76: CACTAA	BC77: AAGCTC	BC78: TACCTT	BC79: CTAGTC	BC80: AATCCG	BC81: GTGTAG	BC82: ACTCTT	BC83: TCAGGA	BC84: ATTGGT
H	BC85: TTGGTA	BC86: CAACAG	BC87: CAATGC	BC88: GGAGGT	BC89: CAGGAC	BC90: GGCCAA	BC91: CTCATA	BC92: CCTGCT	BC93: GGTATA	BC94: TTCCGC	BC95: TAGGCT	BC96: ATATCC

This table is also available in the Application Workbook in Microsoft Excel format (Worksheet *Reagent Plates PrePCR(Sciclone)*, Plate P6).

NOTE: In the 24 reaction SENSE mRNA-Seq V2 kit (Cat. No. 001.24), the columns 4 to 12 of the kit barcode plate are empty.

13. Appendix F: Sequencing*

General

The amount of library loaded onto the flowcell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. From our experience a good starting point is to load between 7 and 14 pM of a SENSE library onto the flowcell. All SENSE libraries can be sequenced using the standard Illumina Multiplexing Read 1, Index read, and Multiplexing Read 2 Sequencing Primers.

External barcodes (6 nt) are introduced during PCR. External barcodes are included in the SENSE mRNA-Seq V2 kits (Cat. No. 001.24 and Cat. No. 001.96) and are provided in a 96-well plate (see also Appendix E, p.35). A schematic representation of those libraries is shown below.

```
                              5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert…

               5'-(Index Read Sequencing Primer)-3'
   …Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-Index-ATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-Index-TAGAGCATACGGCAGAAGACGAAC 5'
                3'-(Read 2 Sequencing Primer)-5'
```

Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Index Read: Multiplexing Index Read Sequencing Primer (not supplied):
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

* Note: Some nucleotide sequences shown in Appendix E may be copyrighted by Illumina, Inc.
Oligonucleotide sequences © 2007-2012 Illumina, Inc.

14. Appendix G: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of SENSE NGS data, and is kept as general as possible for integration with your standard pipeline. In contrast to most other library preparation protocols, SENSE libraries generate reads in a strand orientation opposite to the genomic reference. Reads must be re-oriented during data processing, either by conversion into their reverse complement before mapping or by inverting the directionality flag in the alignment files after mapping.

Processing Raw Reads

We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the dataset.

In order to reduce the bias introduced by the RT and hence to achieve better cluster identification on Illumina platforms, SENSE starters are random nonamer starters.

De-multiplexing

SENSE External Barcodes: The barcode is contained in the Index Read, and demultiplexing can be carried out by the standard Illumina pipeline.

Trimming

As SENSE is based on random priming, there may be a higher proportion of errors at the first nucleotides of the insert due to non-specific hybridization of the starter/stopper heterodimer to the RNA. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner, in which case it may be beneficial to trim these nucleotides. Trimming can be done with the same work-flow for both reads in a paired-end dataset. Please ensure that the selected tool preserves the read-pair information. The first nine nucleotides need to be removed from Read 1 (starter side), while on the stopper side it is only six nucleotides (Read 2).
While trimming the first nucleotides introduced by the starter/stopper can decrease the number of reads of suitable length, the absolute number of mapping reads usually increases due to the improved read quality. Reads which are too short or have generally low quality scores should be removed from the set.

Alignment

At this point the filtered and trimmed reads can be reverse complemented and aligned with a short read aligner to the reference genome or assembled de novo. Alternatively, reads can be mapped first without conversion to the reverse complement, and then the directionality flag in the alignment files can be inverted.

Transcriptome Modeling

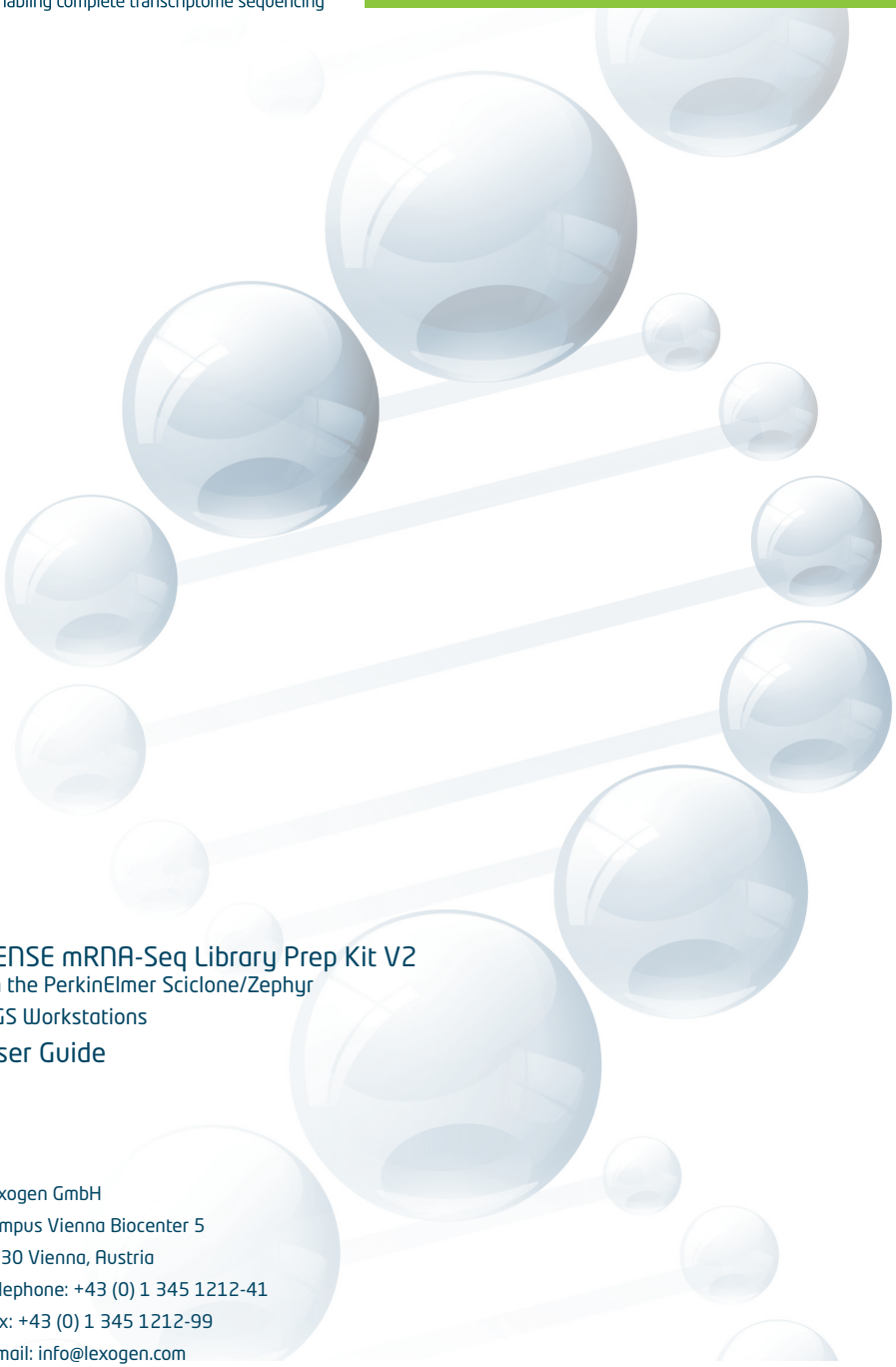
The resulting alignment files are used to model the transcriptome and assess transcript abundance. Further analyses are experiment-specific and can include differential expression, differential splicing, and promoter usage.

15. Appendix H: Revision History

Revision date	Publication No.	Change	Page
April 1st 2015	001UG057V0100	Initial Release autoSENSE mRNA-Seq V2.	

LEXOGEN

Enabling complete transcriptome sequencing



SENSE mRNA-Seq Library Prep Kit V2

on the PerkinElmer Sciclone/Zephyr

NGS Workstations

User Guide

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