TempO-Seq® Assay User Guide

January 15, 2020

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

The TempO-Seq assays and kits are protected by U.S. Patent 9,856,521. Certain kit components are used in the methods of U.S. Patents 9,938,566, 9,957,550, 9,856,521 and UK Patent 2542929. Patents pending in the U.S. and other countries.

For Material Safety Data Sheets (MSDS) and other safety information, contact BioSpyder Technical Support (support@BioSpyder.com).

Contents

Abbreviations

Term	Definition
bp	Base pair
DO	Detector Oligonucleotide (Oligo)
DDO	Down-Stream Detector Oligo. The term “down-stream” refers to its position closer to 3’ end of the target molecule.
FFPE	Formalin-Fixed Paraffin-Embedded
NGS	Next-Generation Sequencing
nt	Nucleotide
Probe	Detector oligo pair that hybridizes to a region of RNA
Target	RNA sequence monitored by hybridizing to detector oligos
TempO-Seq	Templated Oligo Sequencing Assay
UDO	Up-Stream Detector Oligo. The term “up-stream” refers to its position closer to the 5’ end of the mRNA.

Assay Principle

The TempO-Seq assay utilizes a detector oligo (DO) pool specific to the RNA targets to be monitored. BioSpyder offers fixed content and custom DO pools to measure focused sets of transcripts, up to whole transcriptomes. The assay can utilize purified RNA, cell lysates, tissue lysates, or FFPE tissue lysates (FFPE kit available separately). In addition, both positive and negative control samples can be included.

Samples are annealed to a matched pair of detector oligos (DOs), with each pair targeting a single gene. The DO pool targets up to tens of thousands of RNA sequences, depending on the targeted gene content of the assay, but all share the same pair of primer binding sequences (indicated in Fig 1. by the red and yellow sections of each DO). The DOs hybridize to sequences of the targeted RNA molecule such that their respective 3’ and 5’ ends are adjacent. Each UDO has an additional target-specific sequence at their 3’ end that hybridizes to the targeted RNA.

After DO annealing, excess oligos and mis-hybridized or weakly hybridized DOs are removed by nuclease digestion, and the annealed oligos are ligated to form amplifiable templates. The ligated DOs are amplified with one pair of primers (making each PCR reaction single-plex regardless of the number of genes measured), with a different combination of sample index/tag sequences assigned to each sample. The PCR products are pooled together into a sequencing library, which is then purified and run on an Illumina NGS to count the number of ligated detector oligo sequences per sample (ThermoFisher-compatible kit under development). Because TempO-Seq includes an end-point PCR reaction, quantitation of the amplified products before library pooling is not needed. Very low concentration and negative control samples are expected to show low reads in the sequencing data.

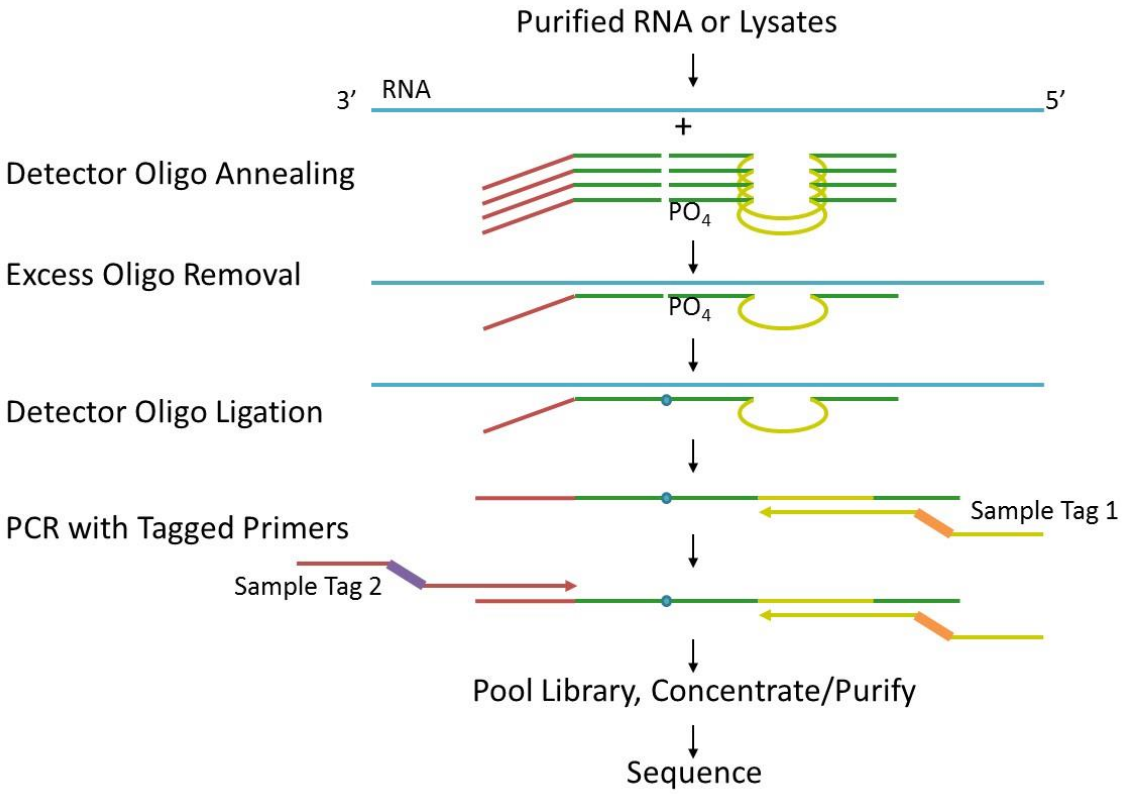


Figure 1: TempO-Seq Biochemistry

The resulting sequencing read counts from the pooled library can be analyzed to determine the abundance of each gene from each sample. Many types of further analysis are then possible; for example, changes in expression due to treatments or differential expression between samples, etc., can be determined.

The TempO-Seq PCR product is structured for single-end (also known as single read) sequencing, with dual-indexing used to identify each sample within the library. The ligated probe sequence is derived from a 50-base read, followed by a 9-base read for Custom Index 1 and a 9-base read for Index 2. Once all 3 sequences are determined, the 50-base read is assigned to the sample that has the corresponding unique combination of index sequences.

Library Sizes

The TempO-Seq Assay currently supports multiplexing of up to 384 samples into a single library for sequencing per lane or flowcell. For smaller libraries, subsets of PCR primers have been separated into smaller kit sizes, designated by a letter (Index Set E, F, G...), each supporting 96 samples. This provides flexibility for making multiple libraries. However, **it is essential to avoid combining samples generated from the same index set**, because different samples will not be distinguishable in the sequencing data if they share the same indices. For example, to prepare a library with 192 samples, you can use two 1 x 96 sample kits with different letter designations,

to assure that the Index Sets are different (e.g. 96 samples processed with Set E PCR primers and the other 96 samples processed with Set F PCR primers, for a total of 192 samples).

For libraries with fewer than 96 samples, kits are available with index primers sufficient for up to 24 or 48 assays. These indices overlap, and thus the 24 or 48 sample PCR primer kits must not be combined with each other, or with samples from larger kits.

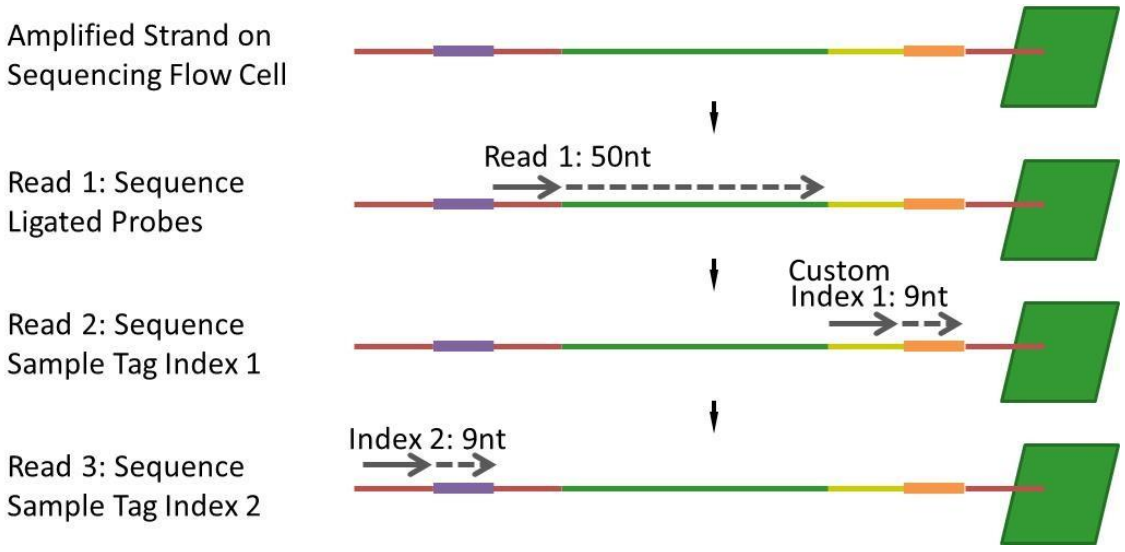


Figure 2: TempO-Seq Sequencing on Illumina NGS Platform.

Experimental Design

The TempO-Seq assay is highly customizable, and allows custom pool generation. Additionally, unlike RNA-seq, the assay allows for true negative control samples, which permit direct measurement of background. We recommend use of technical replicates, positive controls and negative controls in every TempO-Seq experiment. Experimental design support is available from BioSpyder (additional fees may apply). Please contact BioSpyder technical support at support@biospyder.com for details.

TempO-SeqR

To facilitate data processing, analysis, and quality control, BioSpyder has produced an online software package, TempO-SeqR. For more information, visit https://biospyder.com/tempo-seqr/.

TempO-Seq Assay Description

Kit Contents

TempO-Seq assays are available in multiple kit sizes.

Sample Size	Description
1 x 24	Up to 24 samples, PCR primers provided individually.
1 x 48	Up to 48 samples, PCR primers provided individually.
1 x 96	Up to 96 samples, with pre-mixed PCR primers already combined with an amplification mix in 96-well PCR plates, either high or low profile to fit different cyclers.
4 x 96	Up to 384 samples, with pre-mixed PCR primers already combined with an amplification mix in a set of four 96-well PCR plates.

Reagents are shipped frozen, with separate storage temperatures required once they are received. The kits also contain a Custom Index 1 Sequencing Primer for use on the Illumina sequencer when sequencing the TempO-Seq library. **Use of this custom sequencing primer is required.**

TempO-Seq Assay Reagent	Storage Condition
2X Enhanced Lysis Buffer	-20°C
Annealing Buffer	-20°C
DO Pool	-20°C
10X Nuclease Buffer	-20°C
Nuclease	-20°C
10X Ligation Buffer	-20°C
Ligase	-20°C
PCR Pre-Mix (in 24 and 48 sample kits)	-20°C
Forward Primers (in 24 and 48 sample kits)	-20°C
Reverse Primers (in 24 and 48 sample kits)	-20°C
PCR Pre-Mix with Primers Plate (in 96+ sample kits)	-20°C

Additional Materials Provided by the User

Material	Specification
Water	Molecular Biology Grade
Phosphate Buffered Saline (PBS), Ca^{2+} and Mg^{2+} free, Sterile, 1X	Standard PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4, pH 7.4)
NucleoSpin Gel and PCR Clean-up Kit	Macherey-Nagel 740609.50
Pipette Tips	Barrier tips
Assay Plate, 96-well	Microtiter plate, PCR format
PCR Plate, 96-well	
Microtiter plate seals	Adhesive seals
Reagent Reservoir	
Mineral Oil	Molecular Biology grade

All consumables must be RNase and DNase free.

Equipment

Item	Specification
Freezer, -80°C	For RNA and lysate storage
Freezer, -20°C	For reagent storage
Thermocycler	96-well (real-time recommended for PCR step)
Multichannel Pipets	20 µL and 200 µL
Single Channel Pipets	20 µL, 200 µL, and 1000 µL

Access to NGS sequencing instrument or a sequencing provider is required to sequence the library.

Best Practices

The TempO-Seq assay uses RNA within the sample as input and generates a ready-to-sequence library as output. User should be familiar with standard RNA sample processing and protocols to prevent RNA damage and assay contamination with prior amplicons.

To minimize RNA damage before and during the assay, only RNase-free reagents and consumables should be used. Wear gloves and avoid accidental introduction of RNases by keeping containers (assay plates, tip boxes) closed when not in use. Avoid exposing open assay plates or consumables to shed skin cells or aerosols from speaking/coughing/sneezing. Use fresh packages of consumables and use barrier tips.

To minimize amplicon contamination, pre-PCR and post-PCR processes should be physically separated as much as possible. Decontaminate pre-PCR working surfaces with 10% bleach or

1% SDS/0.1 N NaOH. Wear gloves and avoid accidental introduction of amplicons by keeping containers (assay plates, tip boxes) closed when not in use. Avoid transferring amplicons from common areas by replacing gloves after touching handles, phones, computers, etc. Discard or decontaminate anything that falls to the floor.

Vortexing can irreversibly denature proteins, including enzymes, and thus negatively affects assay performance. In steps that contain enzymes (nuclease, ligase, PCR), mix the solution by gently pipetting up and down 3-5 times.

Sample Preparation

TempO-Seq has been successfully used to measure gene expression in a wide variety of samples, including (but not limited to) purifed RNA, cells, fresh or frozen tissues, FFPE tissue, buffy coat leukocytes, PBMCs, CTCs, whole blood, saliva, exosomes, hair follicles, and others. Specific protocols for processing some of these samples are described below. For other sample types, please contact our technical support at support@biospyder.com.

Use only TempO-Seq Enhanced Lysis Buffer for sample preparation. Samples lysed in other buffers are not compatible with the assay.

TempO-Seq Enhanced Lysis Buffer should be kept at -20°C when not in use. Mix well by inversion after thawing. When thawed, the buffer is stable for up to one day at room temperature (25°C), or up to a week at 4°C. For multiple uses, buffer can be re-frozen and re-thawed up to five times. If the buffer is to be used over a long period of time, we recommend aliquoting, freezing, and thawing individual aliquots as needed.

Purified RNA samples

- Purified RNA should be resuspended or diluted in water or Qiagen EB buffer. If the RNA has been purified in another buffer, please contact BioSpyder technical support.
    - Higher input concentration can improve reproducibility, while lower input concentration may increase noise. If the amount of RNA is not limiting, resuspend or elute RNA to a final concentration of 100 ng/µL to maximize the signal to noise ratio. This is especially important if the RNA quality is low. See Appendix A for further discussion on input amounts.
    - If the amount of RNA is limiting, use the following guidelines:
        - For high plexity assays (>5000 probes, such as Whole Transcriptome), RNA should be resuspended to a final concentration of at least 10 ng/µL.
        - For lower plexity assays (<5000 probes, such as TempO-Seq Human Surrogate+Tox Panel 1.1), resuspend RNA to a final concentration of at least 1 ng/µL.
- While taking care to minimize time at room temperature, add an equal volume of 2X TempO-Seq Enhanced Lysis Buffer to the RNA sample. This produces an assay-ready sample in 1X TempO-Seq Enhanced Lysis Buffer.

Lysis of cultured or purified cells

- This protocol applies to both live and 1% PFA-fixed cells.
- The assay can use sample inputs as low as a single cell, and as high as 8 million cells/mL. However, input amounts can affect reproducibility and noise levels. See Appendix A for discussion on how to select the correct input amount for your system.
- For cells that are in suspension
    - Wash cells in PBS.
    - Resuspend cells in PBS. A common range for assay input is 0.5 to 2 million cells per mL. See Appendix A.
    - Add an equal volume of 2X TempO-Seq Enhanced Lysis Buffer to the resuspended cells.
    - Mix to ensure all cells are exposed to the buffer.
    - Incubate the lysates for 10 minutes at 37°C. Do not exceed this incubation time, as lysates may turn mucinous (goopy) and difficult to pipette.
- For adherent cells
    - Prepare sufficient volume of 1X TempO-Seq Enhanced Lysis Buffer, by mixing equal volumes of PBS and 2X TempO-Seq Enhanced Lysis Buffer.
    - Aspirate the media, wash with PBS and aspirate the PBS.
    - Immediately add an amount of 1X TempO-Seq Enhanced Lysis Buffer sufficient to cover the bottom of the well completely and lyse all cells. A common range of concentration for assay input is 0.5 to 2 million cells per mL. See Appendix A.
    - Mix well by nutating or rocking the plate.
    - Incubate the lysates for 10 minutes at 37°C. Do not exceed this incubation time, as lysates may turn mucinous (goopy) and difficult to pipette.
- At this point, lysates are ready for input into the assay. Lysates should be homogeneous before addition to the assay – mix well by pipetting before addition. Avoid creating bubbles during the mixing process.

Optional stopping point: lysates may be frozen before use. Frozen lysates can be stored at -80°C for up to 6 months. Lysates should be thawed at room temperature, immediately before processing through the remainder of the assay. Mix each sample well by pipetting before addition to the assay.

TempO-Seq Assay Protocol

General Notes on the Assay

The TempO-Seq assay process consists of a temperature ramp for DO annealing, constant temperature incubations for nuclease digestion and ligation steps, and a high temperature denaturation step before amplification. It is recommended to pre-program a thermocycler for the steps laid out in the following sections before the assay plate is set up.

Annealing

Prepare the Annealing Mix immediately before starting the assay process. Tables 1A and 1B describe the amounts per sample. The annealing mix should be used within 24 hours of preparation.

If using lower plexity TempO-Seq panels, refer to Table 1A. If using high plexity panels, refer to Table 1B.

Table 1A: Annealing Mix for panels with <5,000 probes

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
5X Annealing Buffer	0.4 µL	11 µL	21 µL	42 µL
Detector Oligo Pool	1 µL	26 µL	53 µL	106 µL
Water	0.6 µL	16 µL	32 µL	63 µL

* Includes 10% overage for pipetting losses.

Table 1B: Annealing Mix for panels with >5,000 probes (e.g. Whole Transcriptome)

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
2X Annealing Buffer	1 µL	26 µL	53 µL	106 µL
Detector Oligo Pool	1 µL	26 µL	53 µL	106 µL

* Includes 10% overage for pipetting losses.

- Mix the components for the appropriate Annealing Mix thoroughly, then aliquot 2 µL into the required number of wells of a 96-well PCR plate or tube strip compatible with the intended thermocycler.
- Transfer 2 µL of sample in 1X TempO-Seq Enhanced Lysis Buffer into the well and mix by gently pipetting the mixure up and down 3-5 times. Avoid creation of bubbles in the mixture.
- If using the mix from Table 1B, overlay the mixture with 6 µL of molecular biology grade mineral oil. This will prevent evaporation during the longer incubation time required for panels with >5000 probes.
- Seal the plate with an adhesive or pressure-sensitive seal.
- Centrifuge briefly and place in the thermocycler.
- **For panels with <5000 probes:** incubate the plate at 70°C for 10 minutes, then ramp to 45°C over 50 minutes, with heated lid. Ramp rate should be as even as possible, ideally

at 0.5°C/minute. Once the plate reaches 45°C for at least 1 minute, remove it to the benchtop (room temperature).

- **For panels with >5000 probes:** incubate the plate at 70°C for 10 minutes, then ramp to 45°C over 50 minutes, with heated lid. Ramp rate should be as even as possible, ideally at 0.5°C/minute. Keep the plate at 45°C for 16 hours, then cool to 25°C. The plate should be taken to the next step of the assay or frozen within 8 hours of reaching 25°C.

Optional stopping point: at this point, the assay plate may be stored frozen overnight at -20°C.

If processing multiple plates, do not stack them on top of each other. The heated top surface of the bottom plate may heat up the wells of the plate stacked on top, causing dissociation of DOs from their target RNAs.

Digestion

Prepare the Nuclease Mix immediately before proceeding. Keep the nuclease at -20°C until use. Tables 2A and 2B describe the amounts per sample. The nuclease mix should be used immediately after preparation.

If using lower plexity TempO-Seq panels, refer to Table 2A. If using high plexity panels, refer to Table 2B.

Table 2A: Nuclease Mix for panels with <5000 probes

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
10X Nuclease Buffer	2.4 µL	63 µL	127 µL	253 µL
Nuclease	0.25 µL	7 µL	13 µL	26 µL
Water	21.4 µL	565 µL	1130 µL	2260 µL

* Includes 10% overage for pipetting losses.

Table 2B: Nuclease Mix for panels with >5000 probes (e.g. Whole Transcriptome)

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
10X Nuclease Buffer	2.4 µL	63 µL	127 µL	253 µL
Nuclease	0.5 µL	13.2 µL	26.4 µL	52.8 µL
Water	21.1 µL	557 µL	1120 µL	2230 µL

* Includes 10% overage for pipetting losses.

- Add 24 µL per sample of prepared Nuclease Mix and mix thoroughly by pipetting.
- Seal the plate with a pressure-sensitive or adhesive seal.
- Centrifuge briefly and place in a thermocycler.
- Incubate the plate for 1.5 hours at 37°C, with heated lid.

Ligation

Prepare the Ligation Mix immediately before starting the assay process. Keep the Ligase at -20°C until use. Table 3 describes the amounts per sample. The Ligase Mix should be used immediately after preparation.

The same Ligase Mix is used for all TempO-Seq panels.

Table 3: Ligation Mix

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
10X Ligation Buffer	2.4 µL	63 µL	127 µL	253 µL
Ligase	1 µL	26 µL	53 µL	106 µL
Water	20.6 µL	544 µL	1090 µL	2180 µL

* Includes 10% overage for pipetting losses.

- Add 24 µL of Ligation Mix to each well, mix by gently pipetting.
- Seal the plate with a pressure-sensitive or adhesive seal.
- Centrifuge briefly and place in a thermocycler.
- Incubate for 1 hour at 37°C, then immediately raise the temperature to 80°C for 15 minutes. Use the heated lid on the thermocycler.
- Remove the plate to a benchtop.

Optional stopping point: the assay plate may be stored frozen at -20°C after this step for up to one week.

Amplification (for 24 or 48 Sample Kits):

- Add 8 µL of PCR Pre-Mix to each well of a PCR plate. The PCR Pre-Mix contains a dsDNA-binding dye, to permit monitoring of assay yield in a green fluorescence channel in a real-time PCR instrument.
- Add 1 µL each of forward and reverse PCR primers per well. Use a separate Forward primer for each row and a separate Reverse primer for each column in the PCR plate.

Do not combine samples from different kits, as the Index Sequences overlap.

- Add 10 µL each of ligated assay product and mix gently by pipetting.
- Seal the plate with a pressure-sensitive or adhesive seal. Alternatively: seal with optical seal for real-time fluorescence detection, if desired.
- Proceed to PCR step.

Amplification (for 96 Sample Kits):

- Briefly centrifuge a 96-well PCR plate containing PCR Pre-Mix and Primers to make sure none of the solution adheres to the plate seal.
- Thaw the plate by letting it stand at room temperature for 10 minutes. The PCR Pre-Mix contains a dsDNA-binding dye, to permit monitoring of assay yield in a green fluorescence channel in a real-time PCR instrument.
- Add 10 µL each of ligated assay product and mix gently by pipetting.
- Optional: if the PCR plate delivered within the kit does not fit your thermocycler, transfer the entire mixture into a compatible plate.

If transferring to a new plate, avoid cross contamination between wells.

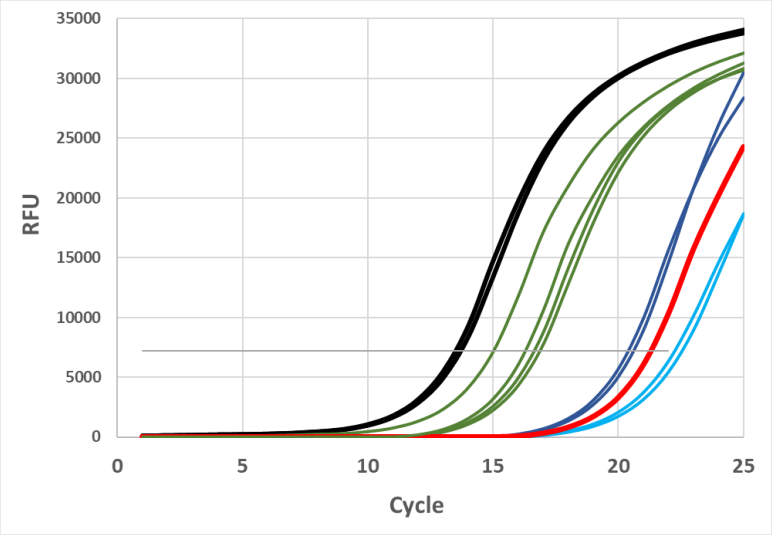
- Seal the plate with a pressure-sensitive or adhesive seal. Alternatively: seal with optical seals for real-time fluorescence detection, if desired. Centrifuge the plate briefly.
- Proceed to PCR step.

PCR

- Cycle as follows.
  1. 37°C 10 minutes
  2. 95°C 1 minute
  3. 25 cycles of 95°C 10 sec; 65°C 30 sec; 68°C 30 sec
  4. 68°C 2 minutes
  5. Hold at 25°C
- For low input samples (lysates with lower than recommended cell concentration, low RNA input, low quality RNA input), or for <500 plexity assays, see Appendix B.
- Optional: if using a real-time PCR instrument, fluorescence reading in green channel can be taken at the end of each cycle in step 3. See next section for details.
- Optional stopping point: PCR plate can be stored at -20°C in a post-PCR freezer for up to one year.

Quality Control

Optional: if a real-time PCR instrument was used for amplification, and if positive and negative control samples were included, it is possible to evaluate the approximate quality of the assay results before they are sent for sequencing. The figure below shows a range of possible results for a TempO-Seq Human Whole Transcriptome assay.



Positive controls (100 ng RNA traces) are shown in black. Negative (no-sample) controls are shown in red. Samples with traces close to the positive control, and well-separated from the no-sample control are likely to produce good results in sequencing (green). Samples that are close to the no-sample controls are likely to produce poor results (dark blue). Samples that amplify after the no-sample control indicate that an inhibitor may be present, such as a large quantity of glycoproteins or DNA in the lysates (light blue). Refer to appendices C and D for advice on cell treatment and troubleshooting. If poor traces persist, please contact BioSpyder technical support (support@biospyder.com).

Library Purification

- Pool 5 µL of each sample in the PCR plate into a microfuge tube or reagent reservoir.
    - Optional step: seal the remaining products in the PCR plate for storage at -20°C in a post-PCR freezer for up to a year.
- Purify the pooled library using the Macherey-Nagel NucleoSpin Gel and PCR Cleanup Kit (catalog number 740609.50 or 740609.250), making the following adjustments to the process in the NucleoSpin User Manual:
    - Wash twice with buffer NT3, wicking any remaining buffer before transferring to an empty collection tube. (Ensure that ethanol has been added to buffer NT3 before first use.)
    - Increase the spin-to-dry step from 1 minute to 10 minutes.
    - Elute twice in NE buffer to maximize yield. Use 15 µL of NE buffer per elution.

Do not forget to add ethanol to buffer NT3 before use.

- Quantitate the purified pooled library according to the sequencing provider's requirements. Expected library concentration should exceed 100 nM.

Sequencing

- The TempO-Seq Assay is validated for use on MiSeq, MiniSeq, NextSeq, HiSeq 2000, and HiSeq 2500. Users unfamiliar with next-generation sequencing should consult Appendix D.
- If available, use 1x50 or 1x75 single-read sequencing kits. However, paired-end flow cells can be used as well.

- Consult with your sequencing service provider for their library and primer requirements.
- Generate sample sheets using TempO-Seq templates from BioSpyder, or by using the Sample Sheet Generator option in TempO-SeqR. Use the appropriate template for the intended sequencing instrument.
- Details for sequencing include the following:
    - The final concentration of the library should be at the upper end of the manufacturer's recommended range.
    - Add 2% PhiX to the library.
    - The library is dual indexed:
        - For Read 1, use standard Illumina Read 1 Primer.
        - For Index 1, **use the TempO-Seq Custom Index 1 Sequencing Primer**, provided in the TempO-Seq Assay kits. Follow Illumina guidelines for custom primer use. Make sure the service provider is aware of this requirement.
        - For Index 2, use the standard Illumina Index 2 Primer (the Illumina P5 primer).
    - If multiple sequencing runs are expected from the same TempO-Seq kit, plan to obtain sufficient amount of the TempO-Seq Custom Index 1 Sequencing Primer (part number 200340).
    - 50 base pairs should be sequenced for Read 1, 9 bp for Index 1 (using BioSpyder Custom Index 1 Sequencing Primer), and 9 bp for Index 2.
    - Do not use adapter filters or trimming, which can remove otherwise legitimate reads.
    - Specify demultiplexed FASTQ files as sequencer output.
    - The amplicon size for TempO-Seq libraries is 187 bp.
- Required sequencing capacity depends on the number of samples in the library and the desired sequencing depth. As a general guideline, follow this formula:

  (number of samples) * (desired total reads per sample) * B = sequencing output

  The factor B incorporates sequencing system inefficiencies. Based on BioSpyder experience, for standard samples, B usually ranges from 1.1 to 1.3.

Certain sequencing instruments can run one library across multiple flowcell lanes, which produces multiple FASTQ files for each sample. See appendix D for details.

Data Analysis

FASTQ files containing sequencing reads can be converted to a read count table using TempO-SeqR software (temposeqr.biospyder.com) or other third-party tools. A manifest of expected sequences for aligning the reads is provided via email with purchase of the assay kits, or by request from technical support (support@biospyder.com).

Appendix A – RNA Input

TempO-Seq can accept a wide range of sample input amounts, from picograms (e.g. single cell) to micrograms (concentrated purified RNA). While all these input levels can produce excellent data, performance in terms of reproducibility, signal/noise ratio, and dynamic range will generally improve with increased RNA input up to the recommended maximum in the protocol.

Figure A1 illustrates the effect of various input levels on the results of the 21,000+ probe Human Whole Transcriptome v1.1 assay. Decreasing total RNA input from 100 ng to 1 ng increases noise among the lower expressing genes, and reduces reproducibility. Decreasing from 10 ng to 1 ng input also reduces the dynamic range of the results. For comparison, the last panel shows the no input negative control sample replicates, indicating the background noise.

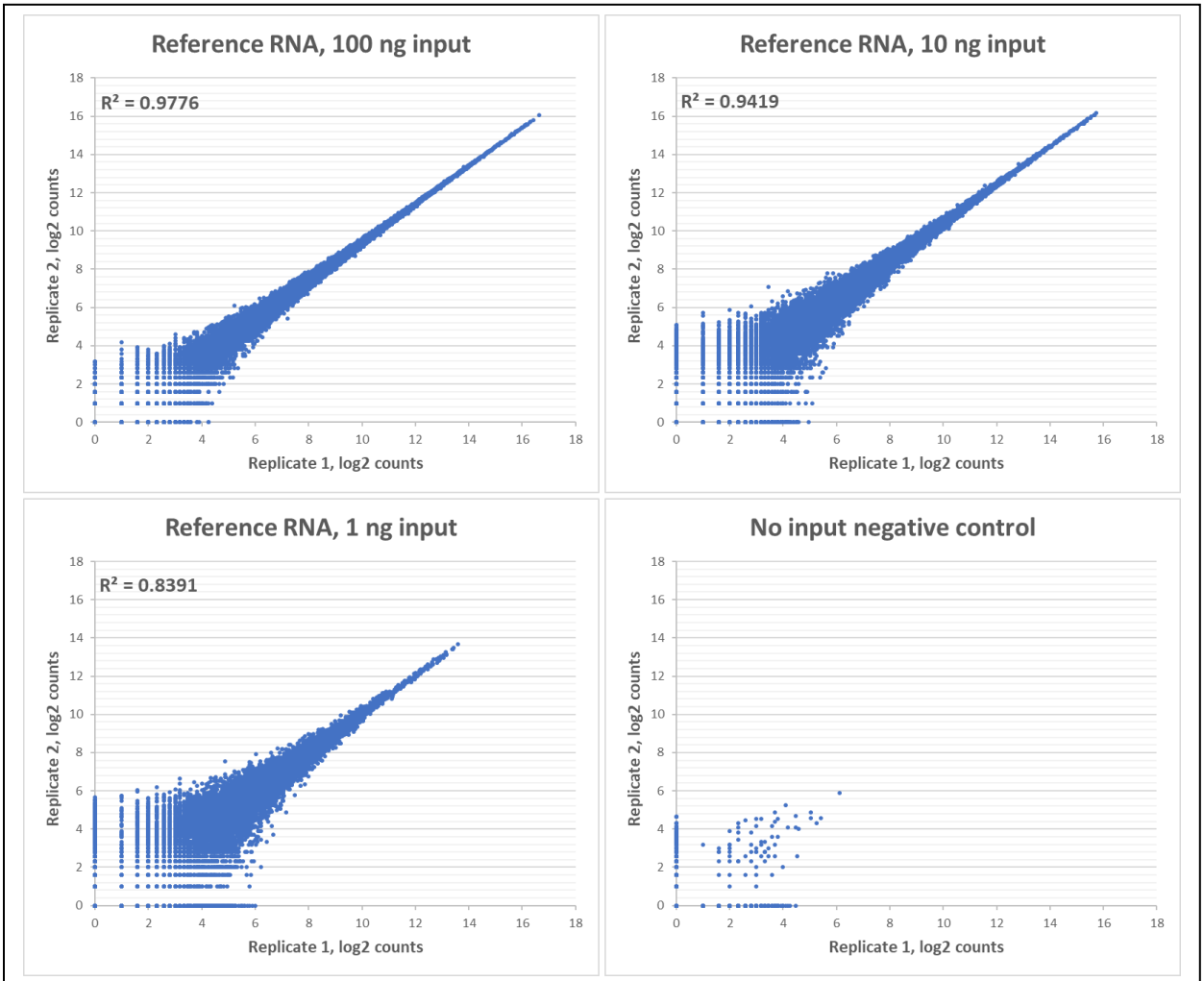


Figure A1: Effect of different RNA input amounts on the results of Human Whole Transcriptome 1.1 assay.

The sensitivity of the assay to input depends on the number of probes present in the panel. Lower plexity assays result in higher reproducibility with much lower inputs. A pilot titration experiment can be used to estimate the optimal input level for a particular system.

In the case of cell lysates, the input issue is complicated by the fact that lysis of too many cells can introduce inhibitory factors (such as proteins, glycans, DNA, and ions). These can interfere with the assay either directly, or by making the lysate muculent and non-homogenous.

For most cell lines, the optimal range for assay input is between 50 and 1,000 cells for panels with <5,000 probes; or between 500 and 2,000 cells for panels with >5,000 probes. This range has been tested on a variety of cell lines, including (but not limited to): MCF7, MDA-MB-231, SK-BR3, K-562, Jurkat, VCAP, HepG2, H4IIE, NRK-52E, HepRG, DU145, A549, 3T3, H2228, HL60, HT29, and PC3.

Some cell lines, especially those with extensive glycoprotein extracellular matrix, produce lysates which are very muculent and difficult to pipet accurately. Increasing incubation time at 37°C after addition of the 2X TempO-Seq Enhanced Lysis Buffer to 30 minutes may help to resolve this problem. We recommend testing a small sample to find the optimal conditions for a particular system.

Lower inputs (e.g. single cells) can lead to increased noise and decreased reproducibility. The principles from the previous discussion of RNA input amount apply to cell lysates (see Figure A2 for an example). We recommend against using more than 2,000 cells per sample.

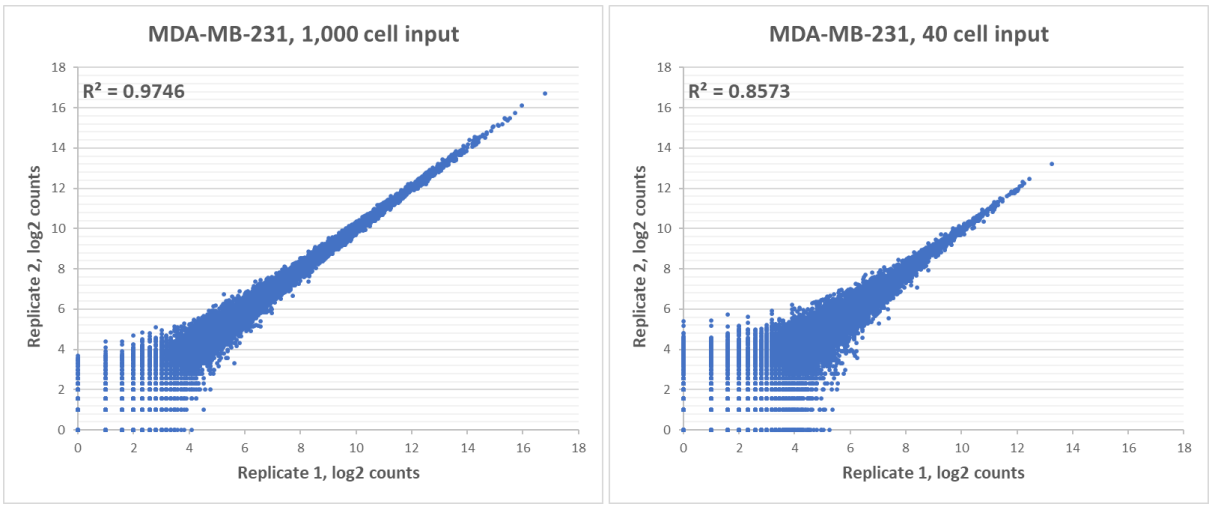


Figure A2: Effects of cell input amount on the results of Human Whole Transcriptome 1.1 assay.

Appendix B – PCR Cycle Number

The PCR step of the assay has been tuned for optimal performance for the majority of sample types and concentrations. It consists of the following steps:

1. 37°C 10 minutes
2. 95°C 1 minute
3. 25 cycles of 95°C 10 sec; 65°C 30 sec; 68°C 30 sec
4. 68°C 2 minutes
5. Hold at 25°C

However, in cases where input is low (few cells, or <5 ng of RNA), or in cases where probe plexity is low (assays with <500 probes), these cycling conditions may produce an insufficient amount of product for successful library preparation. To improve yield in these cases, the number of cycles in step 3 should be increased to greater than 25, as described below. The TempO-Seq PCR reagent includes a dsDNA dye which allows monitoring of product yield in a real-time PCR instrument and optimization of the number of cycles, using the green fluorescence channel.

Figure B1 demonstrates two real-time PCR amplification curves adapted from a 200-probe custom TempO-Seq assay. The black trace shows 10 ng input, while blue traces show <1 ng input. The red trace is a no input negative control sample. The number of cycles on the x-axis refers to step 3 of the PCR. In this experiment, low input samples exhibited no measurable product until cycles 23-28. Increasing the number of cycles in step 4 to 30 was required to generate sufficient material for sequencing. The no input negative control sample shows the point at which nonspecific amplification becomes measurable. If sample traces overlap with the no input control traces, the input amount may be too low regardless of the cycle optimization.

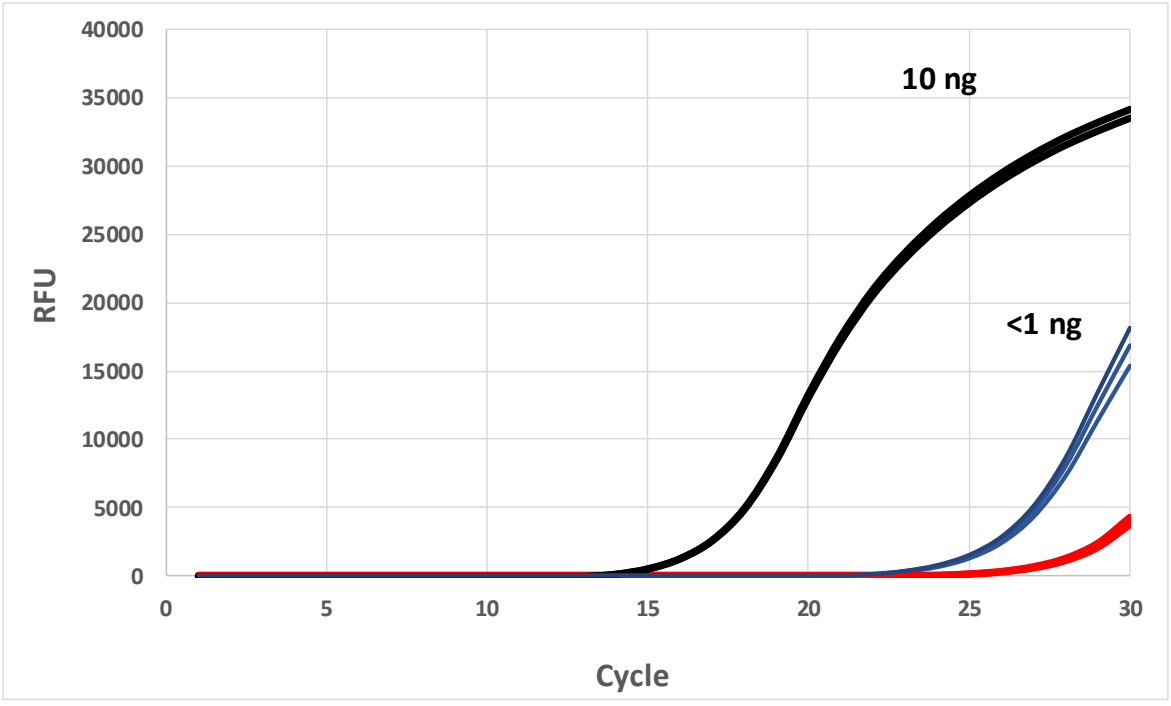


Figure B1: Amplification Traces Depending on Input.

Appendix C – Best Practices for Cell Culture and Lysis

TempO-Seq has been successfully performed on many different cultured cell types and conditions, including primary cell cultures, cells grown on feeder layers, cells grown in/on collagen or Matrigel matrices, IPSCs, and 3D spheroids/organoids/microtissues. While this demonstrates robustness and high resistance to sample variation, additional considerations can minimize potential variability due to culture conditions and maximize resulting data quality.

Cell type. Different cell lines can produce varying amounts of total RNA, therefore impacting the total input (see Appendix A). Furthermore, they can differ in the way they may aggregate or associate with each other, in the type of extracellular matrix they produce, the growth byproducts they exude into the surrounding media, etc.

- Before beginning a large project on a cell type you have not previously used with TempO-Seq, it is recommended to run a small pilot experiment.
    - Wash and lyse a small number of samples (e.g. 3-5) of the new cell type, along with at least two replicates of a negative (no-sample) control.
    - Perform TempO-Seq up to the amplification step for generation of real-time QC data.
    - If the real-time QC traces for the lysates are well separated (>5 Ct difference) from the no-sample control, the larger experiment can proceed.
    - If the real-time QC traces are close to or overlapping with the negative control traces:
        - Optimize cell washing conditions (see below).
        - Increase total input (increase the number of cells or lyse the same number of cells in a smaller volume of lysis buffer).
    - Optimizing washing and input typically resolves the vast majority of problems. If the issue persists, please contact BioSpyder technical support (support@biospyder.com).
- Some cells exude a layer of glycoproteins (e.g. lung epithelial culture), which can then impede the assay. It is imperative to wash such layers away thoroughly before lysing.
- Some cell types will produce extremely mucinous (goopy) lysates due to DNA or other macromolecule-induced clumping. When pipetting such samples into TempO-Seq plates, make sure to avoid transferring the aggregates as much as possible. The DNA will not interfere with the assay, but the volume contains little to no RNA, and thus the sample may fail due to insufficient amount of RNA input. The viscosity can also affect pipetting efficiency, so that less than 2 µL of sample is actually aspirated into the pipette tip; if working with these samples, pay close attention to the volumes being transferred.
- If dealing with very complex culture types (cells on feeder layers, or cells embedded in collagen or other matrices), please contact BioSpyder technical support.

Washing. Some media components and products of cell culture growth can interfere with RNA integrity or with the functional components of the TempO-Seq assay. Of particular note are extracellular RNases produced by many cell types (e.g. hepatocytes), which can damage or destroy RNA released upon cell lysis. Thus, proper washing of cells prior to lysis is essential.

- Only Ca^{2+} and Mg^{2+}-free PBS should be used for cell washing. If this is unfeasible, please contact BioSpyder technical support for advice.
- When washing, make sure that the bottom of the culture plate is completely covered with PBS in each wash step.
- When establishing a washing technique, especially if automation is used, use a microscope to verify that cells are still present in the well after the wash step. Particular care should be taken with cells that associate in 2D, as portions can detach and wash away in sheets.
- Thorough washing is especially important for 3D cultures and organoids, where byproducts and RNases can remain trapped in extracellular spaces.

Lysis. As long as the cells have been washed adequately, lysis problems are rare. However, when dealing with a new cell type or culture conditions:

- Use a microscope to inspect a few wells after addition of TempO-Seq Enhanced Lysis Buffer to verify complete lysis. It is normal to observe some remaining cytoskeletal structures (“cell ghosts”), but most of the cell should dissolve quickly.
- If lysing 3D cultures, additional mixing (trituration) may be required during the 37°C incubation step, or the incubation may need to be prolonged up to 30 minutes. Some remnants of the extracellular matrix may remain, but are unlikely to interfere with the assay.

If you encounter a sample type that strongly resists lysis, please contact BioSpyder technical support for assistance.

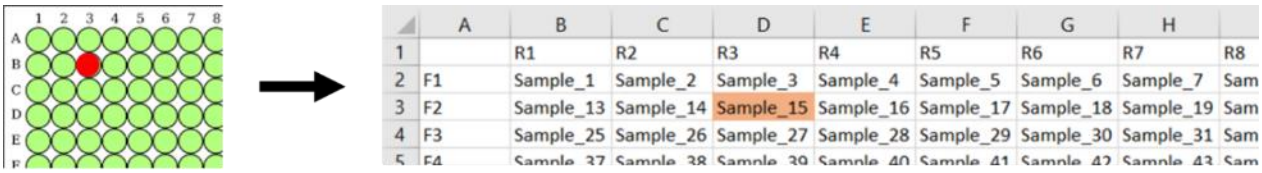
Appendix D – Introduction to Sequencing Data Processing

By utilizing pre-designed probes, TempO-Seq eliminates the need for significant or manual bioinformatics. It is, however, still useful to understand and plan for the processing steps needed to turn a library into a final gene count file. The TempO-SeqR analysis package can be accessed at temposeqr.biospyder.com, and can facilitate most steps of the data flow. Contact support@biospyder.com for information on how to obtain a TempO-SeqR account.

Sample Sheet Generation

Each sample processed through the PCR step of the TempO-Seq assay will have two index sequences added to it. A sample sheet file is needed so that the sequencing instrument will associate individual FASTQ files (and ultimately counts) with the correct samples.

The simplest way to generate a sample sheet is to download a plate template file from TempO-SeqR, and fill the template with sample names (otherwise obtain a sample sheet template from support@biospyder.com and fill it in manually). The sample names must be unique, and can use only letters, numbers, underscores (_), and dashes (-). The figure below illustrates the process: the sample added to well B3 is highlighted. The name for that sample is added to the appropriate cell in a plate template file.



	A	B	C	D	E	F	G	H	
1		R1	R2	R3	R4	R5	R6	R7	R8
2	F1	Sample_1	Sample_2	Sample_3	Sample_4	Sample_5	Sample_6	Sample_7	Sam
3	F2	Sample_13	Sample_14	Sample_15	Sample_16	Sample_17	Sample_18	Sample_19	Sam
4	F3	Sample_25	Sample_26	Sample_27	Sample_28	Sample_29	Sample_30	Sample_31	Sam
5	F4	Sample_37	Sample_38	Sample_39	Sample_40	Sample_41	Sample_42	Sample_43	Sam

Sample_ID	Sample_Name	Sample_Plate	Sample_Well	I7_Index_ID	index	I5_Index_ID	index2	Sample_Project	Description
1	Sample_11	Plate1	A11	R928	ATGTGTGCT	F934	GCAGATGCA	New_Project	control lysate 1
2	Sample_12	Plate1	A12	R928	ATGTGTGCT	F936	AAGTAGGCT	New_Project	control lysate 1
3	Sample_13	Plate1	B1	R928	ATGTGTGCT	F938	ATACGCAGC	New_Project	compound 1 treatment
4	Sample_14	Plate1	B2	R928	ATGTGTGCT	F940	GCACCTATT	New_Project	compound 2 treatment
5	Sample_15	Plate1	B3	R928	ATGTGTGCT	F942	ATACCGGCA	New_Project	compound 3 treatment
6	Sample_16	Plate1	B4	R930	TCGCCACTC	F934	GCAGATGCA	New_Project	compound 4 treatment

When names for all samples have been added, the completed plate template can be uploaded to TempO-SeqR. After selecting which index plate flavor was used, the program will generate the sample sheet that should be submitted along with the library (see figure above, bottom). The sample sheet will contain the sample name you entered, along with the index names and sequences specific to the index plate used. Other information (plate name, project name, description...) can then be filled in manually.

For 24- and 48-sample kits: download the template sample sheet files from the link provided from support@biospyder.com. In this case, the sample names will have to be filled in manually.

Alignment and Data QC

The sequencing instrument will use the information in sample sheet to generate one file for each sample name provided. In most cases, this file will be in FASTQ format. This file is not the final output of the assay, but represents raw sequencing data. Each FASTQ file must be aligned to produce final gene counts.

The easiest way to align your FASTQs is to use the Aligner function of TempO-SeqR. If this is not feasible, users should utilize the R script provided by BioSpyder.

The TempO-SeqR data analysis option can be used to verify data clustering, perform principal component analysis, and verify reproducibility through scatter plots. For more information, consult TempO-SeqR User Guide.

FASTQ Concatenation

Some instruments and configurations produce multiple FASTQ files for the same sample (for example, when the same library is run on multiple lanes of an Illumina HiSeq 2500 flow cell). If these FASTQs are then aligned, the resulting counts for each sample will be divided into multiple columns in the final read count file. There are several ways of dealing with this issue:

- Your sequencing provider or core facility can set the instrument software to automatically concatenate files associated with the same sample name. This is the easiest and most straightforward way.
- Some providers will not concatenate, as it represents an exception to their usual QC and workflow. In these cases, the FASTQ files can still be aligned, and the resulting counts for each sample should be summed to produce final counts.
- If using Unix, FASTQ files for separate lanes can be directly concatenated using the Unix command “zcat.” For example, if Sample_15 results were split into two files, and thus produced two separate FASTQs (labeled L001 and L002), they can be concatenated with the command:

    zcat Sample_15_L001.fastq.gz Sample_15_L002.fastq.gz | gzip > Sample1.fastq.gz

This issue affects only the formatting of the data and ease of its use. The data are not affected or changed by these transformations.

Appendix E – Troubleshooting

Real-time QC traces are flat (including positive controls).

- A key reagent was left out of the assay, or the enzymes used in the assay were improperly stored.

Real-time QC curves for experimental samples have Ct values equal to or greater than those of no-sample controls.

- Low input – too little RNA or cell lysate was added. Verify the concentration of RNA or number of cells/µL of lysate.
- Lysate was not fully transferred into the assay. Check the lysates for "goopy" texture/viscosity, inhomogeneities, pipette-jamming clumps, or other factors that may prevent proper transfer.
- Cells were insufficiently washed before lysis (see Appendix C).
- RNA was degraded before or during the assay. Poor traces from RNA positive control samples will indicate this is a problem. Ensure that proper lab practices are being followed to minimize RNase contamination at all steps of the assay. Use only RNase-free disposables.
- The original sample is high in RNase. If the sample shows poor PCR traces relative to the RNA control, the samples may be high in endogenous RNase. Presence of RNases can be verified by using the purified RNA or lysates as input into the RNase Alert Kit (supplied by Ambion).
- The original sample contains assay inhibitors (such as glycoproteins, hemoglobin, or similar).

The no-sample control real-time QC curve rises very early, overlapping with positive control traces.

- Nuclease Mix was not added to the assay or was incorrectly prepared.
- The nuclease enzyme was improperly stored. Both nuclease and ligase enzymes should be stored at -20°C.
- The no-sample control was contaminated.

Despite real-time QC curves that are well separated from the no-sample control, the purified library yield was lower than expected or has a low 260/230 nm absorbance ratio.

- Ensure that the proper protocol for library purification was followed. Verify that ethanol was added to the column wash buffer.
- Verify that the proper number of cycles was used in PCR (see Appendix B).

The sequencing data did not produce enough reads or the sequencing run failed entirely.

- Verify that you have enough clusters generated in your sequencing run: https://genohub.com/loading-concentrations-optimal-cluster-density/, and that the %Q30 bases are equal to or greater than 85%.
- Verify that your sequencing run was performed on a correct flow cell kit.

- Ensure that 2% Illumina PhiX control was loaded with the library to ensure sufficient sequencing diversity and to help troubleshoot any issues that may have occurred with the sequencing run independent of library preparation.
- Verify that the Custom Index 1 Primer was used, that the sequencing run was set up to use a custom primer, and that the primer was loaded in the correct position according to the instrument documentation.
- Double check that the sample sheet you submitted to your sequencing service provider was accurate and properly formatted. If using the TempO-SeqR sample sheet generator, verify that that proper PCR Pre-mix Index plate flavor was used to generate the sample sheet. If the project included multiple plates, verify that proper plate layouts are associated with the correct flavors.
- The species of the sample does not match the species of the DO pool. Make sure that the FASTQ files are aligned to the correct BioSpyder reference pseudotranscriptome.

Individual samples show high variability in total counts.

- PCR and flow cell binding efficiencies can cause total counts assigned to any given sample to vary up to two-fold.
    - If greater variability is observed, it may be explained by drastic differences in sample input amounts (e.g. some samples at 100 ng of RNA, others at 1 ng). If significantly different types of samples are to be combined in one assay run and cannot be pre-normalized, this variability may be unavoidable, and does not affect the quality of results as long as sufficient depth is generated for all samples.
    - Make sure that all samples are reaching V_{max} during amplification, indicated by real-time PCR QC curves reaching plateau. Final PCR product amounts will be reflected in the number of counts assigned to each sample. Refer to Appendix B for further instructions.

Annealing

Table 1A: Annealing Mix for panels with <5,000 probes

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
5X Annealing Buffer	0.4 µL	11 µL	21 µL	42 µL
Detector Oligo Pool	1 µL	26 µL	53 µL	106 µL
Water	0.6 µL	16 µL	32 µL	63 µL

* Includes 10% overage

- Aliquot 2 µL of the Annealing Mix into a 96-well PCR plate or tube strip.
- Transfer 2 µL of sample in 1X TempO-Seq Enhanced Lysis Buffer into the well, and mix by pipetting.
- Seal the plate.
- Incubate the plate at 70°C for 10 minutes, then ramp to 45°C over 50 minutes. Ramp rate should be as even as possible, ideally at 0.5°C/minute. Once the plate reaches 45°C for at least 1 minute, remove it to room temperature.

If necessary, the assay plate may be stored frozen at this point at -20°C overnight after the annealing step.

Digestion

Table 2: Nuclease Mix for panels with <5,000 probes

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
10X Nuclease Buffer	2.4 µL	63 µL	127 µL	253 µL
Nuclease	0.25 µL	7 µL	13 µL	26 µL
Water	21.4 µL	565 µL	1130 µL	2260 µL

* Includes 10% overage

- Add 24 µL per sample of prepared Nuclease Mix and mix by pipetting.
- Seal the plate.
- Incubate the plate for 1.5 hours at 37°C.

Ligation

Table 3: Ligation Mix

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
10X Ligation Buffer	2.4 µL	63 µL	127 µL	253 µL
Ligase	1 µL	26 µL	53 µL	106 µL
Water	20.6 µL	544 µL	1090 µL	2180 µL

* Includes 10% overage

- Add 24 µL prepared Ligation Mix to each well and mix by pipetting.
- Incubate at 37°C for 1 hour, then immediately at 80°C for 15 minutes.

If necessary, the assay plate may be stored at -20°C for up to a week after this step.

Amplification (for 24 or 48 Sample Kits):

- Add 8 µL of PCR Pre-Mix to each well of a PCR plate.
- Add 1 µL each of forward and reverse PCR primers per well. Use a separate Forward primer for each row and a separate Reverse primer for each column in the PCR plate.

Do not combine samples from the 24 and 48-sample kits, as the Index Sequences overlap.

- Add 10 µL each of ligated sample and mix gently by pipetting.

Amplification (for 96 Sample Kits):

- Thaw a 96-well PCR plate containing PCR Pre-Mix and Primers.
- Briefly centrifuge the plate.

Do not combine more than one plate of the same Index Set in one library.

- Add 10 µL each of ligated sample and mix gently by pipetting.

For All Runs:

- Cycle as follows.
  1. 37°C 10 minutes
  2. 95°C 1 minute
  3. 25 cycles of 95°C 10 sec; 65°C 30 sec; 68°C 30 sec
  4. 68°C 60 sec
  5. Hold at 25°C
- Store PCR plate at -20°C in a post-PCR freezer.

Library Purification

- Pool 5 µL of each sample in the PCR plate into a microfuge tube or reagent reservoir. Optional step: seal the remaining products in the PCR plate for storage at -20°C in a post-PCR freezer.
- Purify the pooled library using the Macherey-Nagel NucleoSpin Gel and PCR Cleanup Kit (catalog number 740609.50 or 740609.250), making the following adjustments to the process in the NucleoSpin User Manual:
  - Wash twice with buffer NT3, wicking any remaining buffer before transferring to an empty collection tube. (Ensure that ethanol has been added to buffer NT3 before first use.)
  - Increase the spin-to-dry step from 1 minute to 10 minutes.
  - Elute twice in NE buffer to maximize yield. Use 15 µL of NE buffer per elution.
- Quantitate the purified pooled library according to the sequencing provider's requirements. Expected library concentration should exceed 100 nM.

Annealing

Table 1A: Annealing Mix for panels with >5,000 probes

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
2X Annealing Buffer	1 µL	26 µL	53 µL	106 µL
Detector Oligo Pool	1 µL	26 µL	53 µL	106 µL

* Includes 10% overage

- Aliquot 2 µL of the Annealing Mix into a 96-well PCR plate or tube strip.
- Transfer 2 µL of sample in 1X TempO-Seq Enhanced Lysis Buffer into the well, and mix by pipetting.
- Overlay the mixture with 6 µL of molecular biology grade mineral oil.
- Seal the plate.
- Incubate the plate at 70°C for 10 minutes, then ramp to 45°C over 50 minutes, with heated lid. Ramp rate should be as even as possible, ideally at 0.5°C/minute. Keep the plate at 45°C for 16 hours, then cool to 25°C. The plate should be taken to the next step of the assay or frozen within 8 hours of reaching 25°C.

If necessary, the assay plate may be stored frozen at this point at -20°C overnight after the annealing step.

Digestion

Table 2: Nuclease Mix for panels with >5,000 probes

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
10X Nuclease Buffer	2.4 µL	63 µL	127 µL	253 µL
Nuclease	0.5 µL	13.2 µL	26.4 µL	52.8 µL
Water	21.1 µL	557 µL	1120 µL	2230 µL

* Includes 10% overage

- Add 24 µL per sample of prepared Nuclease Mix and mix by pipetting.
- Seal the plate.
- Incubate the plate for 1.5 hours at 37°C.

Ligation

Table 3: Ligation Mix

Component	Per Sample	For 24 Samples*	For 48 Samples*	For 96 Samples*
10X Ligation Buffer	2.4 µL	63 µL	127 µL	253 µL
Ligase	1 µL	26 µL	53 µL	106 µL
Water	20.6 µL	544 µL	1090 µL	2180 µL

* Includes 10% overage

- Add 24 µL prepared Ligation Mix to each well and mix by pipetting.
- Incubate at 37°C for 1 hour, then immediately at 80°C for 15 minutes.

If necessary, the assay plate may be stored at -20°C for up to a week after this step.

Amplification (for 24 or 48 Sample Kits):

- Add 8 µL of PCR Pre-Mix to each well of a PCR plate.
- Add 1 µL each of forward and reverse PCR primers per well. Use a separate Forward primer for each row and a separate Reverse primer for each column in the PCR plate.

Do not combine samples from the 24 and 48-sample kits, as the Index Sequences overlap.

- Add 10 µL each of ligated sample and mix gently by pipetting.

Amplification (for 96 Sample Kits):

- Thaw a 96-well PCR plate containing PCR Pre-Mix and Primers.
- Briefly centrifuge the plate.

Do not combine more than one plate of the same Index Set in one library.

- Add 10 µL each of ligated sample and mix gently by pipetting.

For All Runs:

- Cycle as follows.
  6. 37°C 10 minutes
  7. 95°C 1 minute
  8. 25 cycles of 95°C 10 sec; 65°C 30 sec; 68°C 30 sec
  9. 68°C 60 sec
  10. Hold at 25°C
- Store PCR plate at -20°C in a post-PCR freezer.

Library Purification

- Pool 5 µL of each sample in the PCR plate into a microfuge tube or reagent reservoir. Optional step: seal the remaining products in the PCR plate for storage at -20°C in a post-PCR freezer.
- Purify the pooled library using the Macherey-Nagel NucleoSpin Gel and PCR Cleanup Kit (catalog number 740609.50 or 740609.250), making the following adjustments to the process in the NucleoSpin User Manual:
  - Wash twice with buffer NT3, wicking any remaining buffer before transferring to an empty collection tube. (Ensure that ethanol has been added to buffer NT3 before first use.)
  - Increase the spin-to-dry step from 1 minute to 10 minutes.
  - Elute twice in NE buffer to maximize yield. Use 15 µL of NE buffer per elution.
- Quantitate the purified pooled library according to the sequencing provider's requirements. Expected library concentration should exceed 100 nM.