Standard Operating Procedure @ Sciensano and CNR-IREA

PROTOCOL	**TempO-Seq sample preparation**
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1. Purpose

This procedure describes the method for the sample preparation for TempO-Seq transcriptomics analysis.

2. Background

TempO-Seq (Templated Oligo assay with Sequencing readout) is a high-throughput transcriptome analysis method that measures specific gene sequences directly from RNA in crude lysates or purified RNA. The process involves hybridization and ligation of detector oligos (DOs) to target sequences, enzymatic removal of excess DOs, and amplification and sequencing of ligated DO pairs. This method ensures exceptional specificity, even for highly similar genes, by minimizing mis-ligation and background noise, enabling precise and unbiased measurements. It allows for single-base specificity, facilitating the measurement of expressed variants and the differentiation of homologous genes within or between species.

3. Procedure

3.1. Equipments

- Laminar flow cabinet (Technigen or equivalent)
- Cell culture incubator (Thermo Scientific Forma, Model 311 or equivalent)
- -80°C refrigerator (Thermo Scientific, TSE SERIES Model 933 or equivalent)

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165 or Nunclon, with air vent)
- RNAse/DNAse-free cryovial (Smport scientific, T311-1)
- Foil seals (Thermo Fisher Nunc Sealing Tapes, catalog #12-565-398)
- 96-well plate

3.3. Reagents

- PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Biosigma, code: L0615 is stored at 4°C.
- TempO-Seq 2X lysis buffer (Bioclavis) is stored at -20°C
    - For 1X lysis buffer, dilute 2X lysis buffer with equal volume of PBS

Note:

- 2X Enhanced Lysis Buffer should be mixed well after thawing by inverting at least 5 times.
- Once thawed, the buffer is stable for up to one day at room temperature (25°C), or for up to one week at 4°C.

- For multiple uses, 2X Enhanced Lysis Buffer can be re-frozen and thawed up to five times. The buffer must be mixed after each thaw.
- If the buffer is to be used over a long period of time, we recommend aliquoting then freezing and thawing individual aliquots as required. => aliquot in 5 mL aliquots
- Do not use the buffer after the indicated expiration date.
- The lot number of the 2X Enhanced Lysis Buffer used should be recorded on the General Information tab of the Sample Submission Form.

3.4. Experimental procedure

The following procedure has been optimized for HaCaT cells (CLS, Lot. 300493-4820, p32; Eppelheim, Germany). Procedures for cell maintenance at CNR and SC premises are detailed in "SOP_HaCaT cell maintenance_CNR" and "SOP_HaCaT cell maintenance_SC", respectively.

$3x10^5$ cells are seeded in complete medium in 35 mm cell culture dish and the assay is performed after 48 h of growth. Note: the amount of medium in the dishes depends on the optimal field disuniformity in the cell culture dishes and is dependent on the exposure system used (3 ml for CNR and 3.1 ml for SC).

3.4.1. Security

- Protective Clothing: gloves, safety glasses and laboratory coat.
- All manipulations before the exposure of the cells should be performed in a biosafety cabinet. Later manipulations involving chemicals should be performed under a chemical hood.
- All safety measures related to working with potentially carcinogenic chemicals should be respected. Depending on the test products, additional safety measures may be required (see Security sheet).

3.4.2. Controls

- Positive control: no positive control substance will be used.
- Negative control: incubator control, not exposed in either of the two waveguides/reverberation chambers (sham).

3.4.3. Sample preparation

- Start three different cultures at the same time and sub culture separately (for independent experiments).
- Seed three experimental conditions (incubator control, sham exposed and RF exposed) from each culture.

- Label samples per subculture, passage number and exposure condition (or with blinding identifier).
- For the exposures follow the procedure detailed in Annexes 7, 10, 11, 12.
- Prepare sufficient volume of 1X TempO-Seq Enhanced Lysis Buffer
- After the exposure finishes, remove the treatment medium and rinse with 2 mL PBS.
- Immediately add 1 ml 1X TempO-Seq Enhanced Lysis Buffer and mix well by nutating or rocking the plate.
- Incubate the lysates for exactly 15 minutes at 37°C.
- Collect with a tip and transfer to the cryovial.
- Store the samples at -80°C.

Note:

➢ Cell lysate sample requirements: 0.25 to 2 million cells/mL of 1X TempO-Seq Enhanced Lysis Buffer.

3.4.4. Storage and shipping

@ CNR: send samples to Sciensano on dry ice.

@ Sciensano: once all the samples are at SC, gently thaw samples and keep on ice.

- Pipette 60 µl will per sample over to a 96-well plate, according to the Sample submission form, which has to be returned to Bioclavis via e-mail prior to shipping the samples.
- Seal plates to prevent cross-contamination between wells with adhesive aluminium foil film RNAse/DNAse-free.
- Before shipping, inspect the frozen plate assembly to ensure that the seals are secure. Do not wrap plates with Parafilm as it will break into fragments when shipped on dry ice.
- Note plate names (matching those recorded in the Sample Submission Form) on the side of each skirted or semi-skirted plate.
- Freeze plates horizontally so that all liquid freezes at the bottom of the wells. If samples are not at the bottom of the well after sealing the plate, centrifuge briefly.
- Send samples on dry ice to Bioclavis for the analysis

3.4.5. Sample analysis

The TempO-Seq assay is outsourced to Bioclavis, a spinout of BioSpyder Technologies, which specializes in TempO-Seq Analysis. Samples are sent on dry ice to Bioclavis, where synthetic DNA probes complementary to target mRNA transcripts are added to the thawed cell lysates. These probes contain universal primer sites for subsequent amplification.

After the assay is performed by Bioclavis, the data from the sequencing reads will be sent to Sciensano for data analysis. The sequencing reads derived from the TempO-Seq libraries represent the mRNA transcripts captured and labeled during the assay. Bioinformatic analysis of the sequencing data involves quantifying gene expression levels, identifying differentially expressed genes, and inferring biological pathways or functional annotations. The data are normalized and statistically analyzed to generate meaningful insights into gene expression patterns.