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Purification of Long Non-coding RNAs on Replication Forks Using iROND (Isolate RNAs on Nascent DNA)

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Abstract

Fork stability is key to genome DNA duplication and genetic integrity. Long non-coding RNAs (LncRNAs) may play vital roles in fork stabilization and chromatin remodeling. Existing techniques such as NCC-RNA sequencing are useful to identify LncRNAs on nascent chromatin DNA. However, there is still a lack of methods for LncRNAs purification directly from replicative forks, hindering a deep understanding of the functions of LncRNAs in fork regulation. Here, we provide a step-by-step protocol named iROND (isolate RNAs on nascent DNA). iROND was developed and modified from iPOND, a well-known method for purifying fork-associated proteins. iROND relies on click chemistry reaction of 5'-ethynyl-2'-deoxyuridine (EdU)-labeled forks and biotin. After streptavidin pull down, fork-associated LncRNAs and proteins are purified simultaneously. iROND is compatible with downstream RNA sequencing, qPCR confirmation, and immunoblotting. Integrated with functional methods such as RNA fluorescent in situ hybridization (RNA FISH) and DNA fiber assay, it is feasible to screen fork-binding LncRNAs in defined cell lines and explore their functions. In summary, we provide a purification pipeline of fork-associated LncRNAs. iROND is also useful for studying other types of fork-associated non-coding RNAs.

Key features

- Purify long non-coding RNAs (LncRNAs) directly from replication forks.
- Connects to RNA sequencing for screening easily.
- Allows testing various genotoxic stress responses.
- Provides LncRNA candidate list for downstream functional research.

Keywords: LncRNA, Purification, Replication fork, Genotoxic stress, Genomic stability

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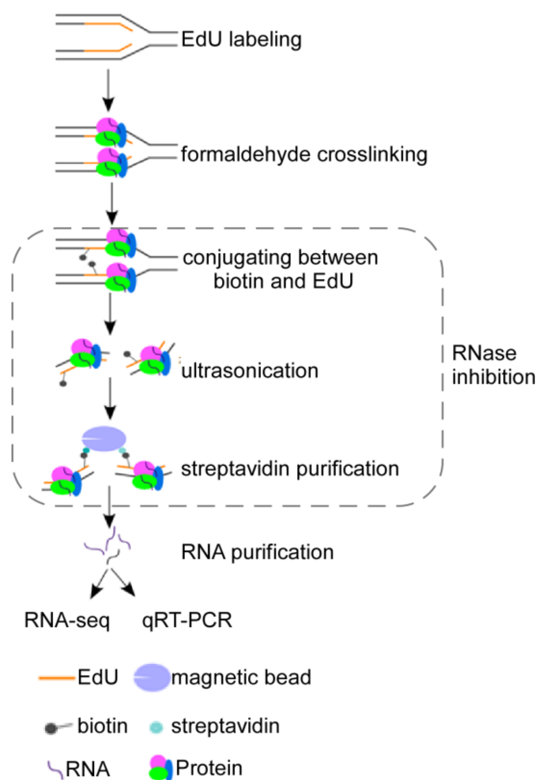
Graphical overview



Schematic overview of isolate RNAs on nascent DNA (iROND) protocol. Cells were pulse-labeled with 5'-ethynyl-2'-deoxyuridine (EdU) for 10 min before paraformaldehyde fixation. EdU-positive forks were ligated with biotin through Click-IT chemistry reaction. Genomic DNA was ultrasonically cracked and crosslinked with streptavidin for pulling down. Both RNA and protein components were purified. RNA components were used for downstream RNA sequencing and qPCR validation. Protein components were used for immunoblotting to evaluate binding dynamics of fork-associated proteins such as helicase, topoisomerase, and DNA polymerases.

Background

DNA replication and DNA repair are central topics in genome stability research. Currently, a vast majority of studies focuses on protein components intensively. Through the investigation of core factors such as ATM, ATR, CHK1, CHK2, DNA-PK, and P53, as well as their associated signaling pathways, the fundamental regulatory mechanisms in DNA replication and DNA repair have been revealed (Jackson and Bartek, 2009). For example, using isolate proteins on nascent DNA (iPOND) technology, numerous replication fork-associated protein components have been identified, including DNA polymerases, helicases, and epigenetic modifiers (Sirbu et al., 2012). The initiation, elongation, and termination of steady-state replication forks are strictly controlled to ensure the integrity of the genome replication process (Dungrawala and Cortez, 2015). In order to cope with genotoxic drug-induced fork collapse or stalling, endogenous DNA replication stress response mechanisms can ensure DNA replication progression and reduce the accumulation of DNA breaks (Berti et al., 2020). In terms of DNA repair, numerous specific techniques have been developed. For instance, GFP-based reporter systems were used to evaluate DNA repairing efficiency (Pierce et al., 1999). Single-cell comet assay is used broadly to examine general DNA damage levels (Collins, 2004). Overall, the involvement of protein components in genome stability maintenance is presently well understood.

Besides protein components, a few recent studies raised the potential importance of RNA components in DNA repair and replication, facilitated by indirect identification techniques. For example, the NCC-RNA-seq technique, based on the principle of biotin affinity purification, has been used to identify multiple long and short RNA molecules associated with nascent chromosomes (Gylling et al., 2020). The functions of these RNAs in chromosome reshaping and maturation warrant further investigation. Additionally, using RNA affinity purification coupled with mass spectrometry, the long non-coding RNA (LncRNA) *NORAD* plays a critical role in genome stability through interaction with PUMILIO protein (Elguindy and Mendell, 2021). Comparative transcriptomics is an effective approach for discovering functional non-coding RNAs. In our previous studies, we identified a novel LncRNA, *DISCN,* by comparing the expression profiles of LncRNAs in mouse embryonic stem cells (mESCs) with differentiated cells. *DISCN* exhibits specific high expression in embryonic and neural stem cells. *DISCN* forms a functional complex with the nucleolar protein nucleolin and the DNA single-strand binding protein RPA, through which it keeps an RPA protein pool to ensure the efficiency of DNA replication stress response and DNA repair (Wang et al., 2021). These studies support the notion that non-coding RNAs form a new regulatory layer in genomic stability system. However, limited by direct purification techniques, the roles of non-coding RNAs in DNA replication or DNA repair are still not understood.

We have recently developed a novel method called iROND (isolate RNAs on nascent DNA). Using iROND, we identified a specific fork-binding LncRNA *Lnc956,* in the embryonic stem cell system. *Lnc956* forms a stable nucleic acid–protein complex with TRIM28 and HSP90B1, facilitating the integration of the CMG helicase at replication fork sites and ensuring replication fork stability in mESCs (Zhang et al., 2023). Here, we provide a step-by-step protocol for iROND. iROND is applicable in different types of rapidly proliferating cell lines and compatible with downstream functional validation systems such as RNA fluorescence in situ hybridization (RNA-FISH) and DNA fiber assays.

Materials and reagents

Biological materials

1. Mouse embryonic stem cells (derived from C57Bl/6J blastocysts)
2. Mouse NIH3T3 cells (kindly provided by Dr. Bingyu Mao at Kunming Institute of Zoology, Chinese Academy of Sciences)

Materials

1. 35 mm cell culture dish (Corning, catalog number: 430165)
2. 60 mm cell culture dish (NEST, catalog number: 705001)
3. 100 mm culture dish (NEST, catalog number: 704001)
4. 150 mm culture dish (Corning, catalog number: 430599)
5. 200 μL PCR tube (Axygen, catalog number: AXYPCR02LC)
6. 1.5 mL centrifugal tube (Axygen, catalog number: AXYMCT150C)
7. 15 mL centrifugal tube (NEST, catalog number: 601052)
8. 50 mL centrifugal tube (NEST, catalog number: 602002)
9. 0.22 μm filter (Millipore, catalog number: SLGP033RB)

Reagents

1. 5-ethynyl-2′-deoxyuridine (EdU) (Life Technology, catalog number: A10044)
2. Thymidine (Sigma-Aldrich, catalog number: T1895)
3. 37% (w/v) formaldehyde solution (Sigma-Aldrich, catalog number: F1635); caution: toxic
4. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
5. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P3911)
6. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)

7. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: S5136)
8. Glycine (Sigma-Aldrich, catalog number: G7126)
9. Triton X-100 (Sigma-Aldrich, catalog number: X100)
10. BSA (Sigma-Aldrich, catalog number: V900933)
11. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)
12. Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) (Sigma-Aldrich, catalog number: 209198)
13. (+) Sodium L-ascorbate (Sigma-Aldrich, catalog number: A4034)
14. Biotin azide (Invitrogen, catalog number: B10184)
15. SDS (Sangon Biotech, catalog number: A600485)
16. Tris (Sangon Biotech, catalog number: A610195)
17. Glycerol (Sangon Biotech, catalog number: A600232)
18. Bromophenol blue (Sangon Biotech, catalog number: A602230)
19. EDTA (Sigma-Aldrich, catalog number: E9884)
20. Agarose (Merck, catalog number: A4718)
21. Dithiothreitol (DTT) (Merck, catalog number: D8255)
22. Aprotinin (Merck, catalog number: A6279)
23. Leupeptin (Merck, catalog number: L2884)
24. Streptavidin agarose (Novagen, catalog number: 69203-3)
25. RNase inhibitor (Thermo Fisher, catalog number: EO0381)
26. Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich, catalog number: D5758)
27. Protease K (Thermo Fisher, catalog number: 4333793)
28. Chloroform (Sigma-Aldrich, catalog number: 366911)
29. Isopropyl alcohol (Sigma-Aldrich, catalog number: W292912)
30. Ammonium acetate (Sigma-Aldrich, catalog number: A7262)
31. Alcohol (Sigma-Aldrich, catalog number: AX0442)
32. Acetic acid (Sigma-Aldrich, catalog number: 695092)
33. TRIzol (Thermo Fisher, catalog number: 15596026)
34. Hydroxyurea (HU) (Sigma-Aldrich, catalog number: H8627)

Solutions

1. EdU solution (see Recipes)
2. DEPC-H_2O (see Recipes)
3. Thymidine solution (see Recipes)
4. Chase medium (see Recipes)
5. 1× PBS solution (see Recipes)
6. 1% Formaldehyde solution (see Recipes)
7. 1.25 M Glycine solution (see Recipes)
8. Permeabilization buffer (see Recipes)
9. Wash buffer (see Recipes)
10. Biotin-azide solution (see Recipes)
11. 100 mM $CuSO_4$ solution (see Recipes)
12. Sodium L-ascorbate solution (see Recipes)
13. Lysis buffer (pH 8.0) (see Recipes)
14. Salt wash buffer (see Recipes)
15. 2× SDS Laemmli sample buffer (2× SB) (see Recipes)
16. 50× Tris acetate-EDTA (TAE) buffer (see Recipes)

Recipes

1. EdU solution

Reagent (use for storing)	Final concentration	Quantity
EdU (50 mg)	10 mM	25.2 mg
DMSO	n/a	10 mL
Total	n/a	10 mL

Reagent (use for cell labeling)	Final concentration	Quantity
EdU (10 mM)	10 µM	1 µL
mESCs medium (see Step A1)	n/a	999 µL
Total	n/a	1 mL

2. **DEPC-H_2O**

Reagent	Final concentration	Quantity
DEPC	n/a	1 mL
H_2O	n/a	999 mL
Total	n/a	1,000 mL

3. **Thymidine solution**

Reagent (use for storing)	Final concentration	Quantity
Thymidine (1 g)	10 mM	24.2 mg
DEPC-H_2O	n/a	10 mL
Total	n/a	10 mL

4. **Chase medium**

Reagent (use for cell labeling)	Final concentration	Quantity
Thymidine (10 mM)	10 µM	1 µL
mESCs Medium	n/a	999 µL
Total	n/a	1 mL

5. **1× PBS solution**

Reagent	Final concentration	Quantity
NaCl	137 mM	8 g
KCl	3 mM	0.2 g
Na_2HPO_4	8 mM	1.15 g
KH_2PO_4	2 mM	0.24 g
DEPC-H_2O	n/a	1 L
Total	n/a	1 L

6. **1% Formaldehyde solution**

Reagent	Final concentration	Quantity
Formaldehyde (37%)	1%	0.27 mL
DEPC-PBS	n/a	9.73 mL
Total	n/a	10 mL

7. **1.25 M Glycine solution**

Reagen	Final concentration	Quantity
Glycine	1.25 M	46.92 g
DEPC-H_2O	n/a	500 mL
Total	n/a	500 mL

8. **Permeabilization buffer**

Reagent	Final concentration	Quantity
Triton X-100	0.25%	2.5 mL
DEPC-PBS	n/a	997.5 mL
Total	n/a	1 L

9. **Wash buffer**

Reagent	Final concentration	Quantity
BSA	0.5%	0.5 g
DEPC-PBS	n/a	100 mL
Total	n/a	100 mL

10. **Biotin-azide solution**

Reagent	Final concentration	Quantity
Biotin-azide	1 mM	1 mg
DMSO	n/a	1.624 mL
Total	n/a	1.624 mL

11. **100 mM $CuSO_4$ solution**

Reagent	Final concentration	Quantity
$CuSO_4 \cdot 5H_2O$	100 mM	1.248 g
DEPC-H_2O	n/a	50 mL
Total	n/a	50 mL

12. **Sodium L-ascorbate solution**

Reagent	Final concentration	Quantity
(+) sodium L-ascorbate	100 mM	20 mg
DEPC-H_2O	n/a	1 mL
Total	n/a	1 mL

13. **Lysis buffer (pH 8.0)**

Reagen	Final concentration	Quantity
Tris	10 mM	1.21 g
SDS	7 mM	2 g
DEPC-H_2O	n/a	200 mL
RNase inhibitor	10 U/μL	200 μg
Aprotinin	1 μg/mL	200 μg
Leupeptin	1 μg/mL	200 μg
Total	n/a	200 mL

14. **Salt wash buffer**

Reagent	Final concentration	Quantity
NaCl	1 M	14.625 g
DEPC-H_2O	n/a	n/a
Total	n/a	250 mL

15. **2× SDS Laemmli sample buffer (2× SB)**

Reagent	Final concentration	Quantity
SDS	123 mM	0.4 g
Bromophenol blue	1 mM	0.01 g
Glycerol	18%	2 mL

1 M Tris (pH 6.8)	1 mM	1.25 mL
1M DTT	1 mM	1.25 mL
H_2O	n/a	8 mL
Total	n/a	12.5 mL

16. 50× Tris acetate-EDTA (TAE) buffer

Reagent	Final concentration	Quantity
Tris	2 M	242 g
Acetic acid	n/a	57.1 mL
EDTA	0.1 M	37.2 g
H_2O	n/a	942.9 mL
Total	n/a	1,000 mL

Equipment

1. Thermo Forma series II water jacketed CO_2 incubator (Thermo Scientific, model: 3111)
2. Nikon eclipse Ti inverted microscope (Nikon, model: Ti)
3. Ultrasonic cell disruptor (Diagenode, Bioruptor Plus)
4. 90 μm nylon mesh (Small Parts Inc., catalog number: B000FN0PGQ)
5. Roll shaker for incubation (Thermo Scientific, model: PHMT)
6. Real-time fluorescence quantitative PCR instrument (Bio-Rad, model: CFX96Touch)
7. Vortexer (VWR analog vortex mixer, catalog number: 10153-838)
8. 4 °C refrigerator (Haier, model: HYC-1090)
9. -20 °C freezer (Haier, model: DWL262)
10. NanoDrop (Thermo Scientific, ND2000)
11. -80 °C freezer (Thermo Scientific, model: TSX60086VRAKLB)

Software

1. Microsoft Excel (Microsoft, https://products.office.com/en-us/excel) (Office Excel 2016, September 22, 2015)
2. GraphPad Prism (GraphPad, https://www.graphpad.com/scientific-software/prism/) (GraphPad Prism 8.3.0, October 29, 2019)

Procedure

A. Label cells with EdU

1. For the preparation of mESC medium and culture and expansion of mESCs and NIH3T3, please refer to our publication (Zhang et al., 2023). Before EdU labeling, incubate cells with 37 °C pre-warmed fresh mESCs medium for 2 h, with 25 mL of medium for each 15 cm diameter dish containing ~1.5×10^7 cells.
2. To perform EdU labeling, take the dishes to a biological safety cabinet, add 25 μL of EdU stock (10 mM) into 25 mL of pre-existing medium for each dish, and mix well gently to make sure that the final concentration of EdU is 10 μM. Depending on the percentage of mESCs in S phase (more than 60%), we calculated that at least 9×10^9 cells in each dish were labeled by EdU.
3. Put the dishes back into the incubator quickly and label for 10 min.

Note: Typically, fork-associated RNA components display a low-dose manner. To improve the productivity, using a cell line with high percentage of S-phase cells is critical to secure the RNA output. In our study, we used mESCs, as these always keep more than 60% of cells in S phase. If you need to evaluate replication stress dynamics, add genotoxic drugs such as 4 mM hydroxyurea (HU) into the dish and incubate for 2 h.

4. To get post-replicated chromatin samples, remove EdU-positive medium, carefully wash cells three times with 5 mL each time of pre-warmed chase medium. Chase medium, containing 10 μM thymidine, is used to compete with EdU to chase DNA replication. After 1 h incubation, the EdU-labeled DNA has been constructed to mature chromatin. So, this group is used as a chromatin control. Then, add 25 mL of pre-warmed chase medium and incubate for 1 h.

B. Formaldehyde crosslink and harvest cells

1. Once finished with EdU labeling, thymidine chase, or drugs treatment, remove culture medium quickly and add 10 mL of ice-cold PBS containing 1% formaldehyde to each dish to fix cells for 20 min at room temperature.
   Caution: Formaldehyde is toxic to human health.
   Critical: Do not wash cells with PBS buffer before fixation since PBS washing may cause RNA degradation.
2. Add 1 mL of 1.25 M glycine to each dish to quench formaldehyde.
3. Scrape cells and harvest into 50 mL RNase-free centrifuge tubes. Record the fixation volume.
   Critical: Keep tubes on ice all the time.
4. Spin down at 900× *g* for 5 min at 4 °C.
5. Remove supernatant.
6. Use ice-cold 1× PBS buffer containing 10 U/μL RNase inhibitor to wash sediments three times with the same volume as the fixation volume from step B3.
   Pause point: Samples can be quickly immersed into liquid nitrogen and stored at -80 °C for at least four weeks.

C. Cell permeabilization

1. Resuspend cells in ice-cold permeabilization buffer containing 10 U/μL RNase inhibitor. Adjust cell density to 1×10^7 cells/mL. Vortex well and incubate at room temperature for 30 min on a shaker (40 rpm).
2. Centrifuge at 900× *g* for 5 min at 4 °C.
3. Remove supernatant.
4. Wash cells with ice-cold 1× PBS containing 0.5% BSA and 10 U/μL RNase inhibitor; the volume used is equal to the permeabilization buffer in step C1.
5. Centrifuge at 900× *g* for 5 min at 4 °C and remove supernatant.
6. Repeat steps C4 and C5 once. Keep samples on ice for click reaction.

D. Click reaction

1. Prepare click reaction cocktail as listed in Table 1.

Table 1. Click reaction cocktail (5 mL for 1×10^8 cells)

Reagent	Stock concentration	Working concentration	Control reaction volume (mL)	Experimental reaction volume (mL)
1× PBS			4.225	4.225
Biotin-azide	1 mM	10 μM		0.05
$CuSO_4$	100 mM	2 mM	0.1	0.1

(+) Sodium L-ascorbate	100 mM	10 mM	0.5	0.5
DMSO			0.05	
RNase inhibitor	40 U/μL	1 U/μL	0.125	0.125
Total volume			5.0	5.0

Critical: The biotin-azide is sensitive to light. The reaction cocktail should be stored in the dark.
Critical: Cu^{II} is unstable in water. The reaction cocktail should be prepared fresh.

2. Resuspend cells into click reaction cocktail. Adjust reaction volume to reach the density of 1×10^8 cells per 5 mL.
3. Vortex gently and incubate for 2 h at room temperature on a shaker (40 rpm).
   Critical: Keep the reaction tubes in the dark.
4. Centrifuge at 900× *g* for 5 min at 4 °C and remove supernatant.
5. Wash cells with ice-cold 1× PBS buffer containing 0.5% BSA and 10 U/μL RNase inhibitor. The PBS volume used is equal to the permeabilization buffer in step C1.
6. Centrifuge at 900× *g* for 5 min at 4 °C and remove supernatant.
7. Repeat steps D5 and D6 once.

E. Cell lysis and sonication

1. Keep lysis buffer on ice. Add leupeptin, aprotinin, and RNase inhibitor before use.
2. Add 100 μL of lysis buffer per 1.5×10^7 cells and resuspend well. Transfer cell suspension into a 1.5 mL RNase-free centrifuge tube.
3. Sonicate cells using a microtip sonicator with the following settings: pulse, 20 s; 40 s pause; 15 cycles; power: 13–16 watts.
   Critical: Tubes should be immersed into an ice-cold water bath in the process of sonication to prevent overheating.
   Note: After sonication, the supernatant should be clear, but not cloudy.
4. Centrifuge at 12,000× *g* for 15 min at 4 °C.
5. Transfer the supernatant through a 90 μm nylon mesh and collect into a new 1.5 mL RNase-free centrifuge tube; then, keep samples on ice.
   Note: To examine DNA fragment size at this step, pick up 5 μL of supernatant to extract DNA. Perform electrophoresis using 1% agarose gel at 120 V for 30 min to detect DNA fragments (Figure 1).

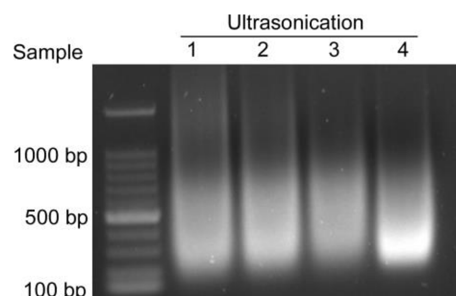


Figure 1. DNA fragments after sonication. The size of DNA fragments is mainly concentrated in the 200–1,000 base pair range.

6. DNA extraction.
   a. Take 5 μL from the supernatant from step E5, add protease K to the final concentration of 50–100 μg/mL, mix well, and incubate at 50 °C for 3 h.

b. Add an equal volume of chloroform/isopropyl alcohol (volume ratio: 24:1), mix well, centrifuge at 10,000× *g* for 10 min, and transfer the supernatant to the new centrifuge tube.
c. Add 10% volume of 10 M ammonium acetate and mix gently.
d. Add twice the volume of isopropyl alcohol and mix gently.
e. Centrifuge at 10,000× *g* for 10 min and discard supernatant.
f. Add the same volume of 75% ethanol and mix well, centrifuge at 10,000× *g* for 5 min, and discard the supernatant.
g. Air-dry pellets for 5 min and dissolve with 10 µL of double-distilled water for electrophoresis.

7. Dilute the samples with the same volume of ice-cold PBS to improve the efficiency of following Biotin capture. Make sure to add 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 10 U/µL RNase inhibitor.
8. Pick up 15 µL of each sample, mix well with 15 µL of 2× SB, and boil at 95 °C for 10 min. These samples can be used as input for immunoblotting targeting replication fork proteins such as PCNA.
Critical: This step is crucial since immunoblotting of PCNA can be used to confirm the pull-down efficiency and specificity.
9. Pick up 15 µL of each sample, mix well with 500 µL of TRIzol, and store at -80 °C for RNA purification.
Critical: This step is crucial; samples will be used to calculate enrichment fold change in the following qPCR confirmation.

F. Streptavidin capture

1. Vortex streptavidin agarose beads well.
2. Calculate agarose beads volume for each sample. Typically, 100 µL of streptavidin agarose beads is used per 1×10^8 cells.
3. Wash streptavidin agarose beads two times with lysis buffer and one time with PBS before use, using 1 mL for each wash.
4. Add beads into each sample and incubate for 16 h at 4 °C on a rotator (40 rpm).
Critical: Keep the rotator in the dark.
5. Centrifuge at 1,800× *g* for 1 min at 4 °C and remove supernatant.
6. Wash beads three times with ice-cold lysis buffer and one time with 1 mL of salt wash buffer. For each wash, samples should be rotated for 5 min before centrifugation.

G. Elution of proteins and RNAs

1. Separate the beads of each sample into two parts equally to elution protein and RNA components, respectively.
2. Add 2 µL of 2× SB into one part, mix well, and boil samples at 95 °C for 25 min to elute proteins. These are ready to use for immunoblotting.
3. For elution of RNAs, add 500 µL of TRIzol into another part of each sample, and mix well.
4. Add 100 µL of chloroform into each tube, vortex tightly for 30 s, and leave on ice for 5 min.
5. Centrifuge at 12,000× *g* for 10 min at 4 °C.
2. Transfer the supernatant of each sample into a new RNase-free tube and record the volume.
3. Add an equal volume of isopropanol and leave on ice for 10 min.
4. Centrifuge at 12,000× *g* for 10 min at 4 °C. Remove the supernatant.
5. Wash the pellet with 75% alcohol.
6. Centrifuge at 12,000× *g* for 10 min at 4 °C. Remove the supernatant and air dry for 1 min.
7. Add 40 µL of RNase-free double-distilled water to dissolve the pellet.
8. Measure RNA concentration using a NanoDrop. Approximately 200–400 ng of RNA can be extracted from 1×10^8 cells.
Note: RNA purification is critically important; the optical density (OD) 260/230 value should be between 1.5 and 2.4, and the OD260/280 value must be between 1.8 and 2.4.

Data analysis

1. Immunoblotting is useful to confirm the pull-down specification of fork DNA fragments. PCNA and Histone H3 can be used as a fork-binding protein marker and mature chromatin marker, respectively. Please refer to Supplementary Figure 1A in our publication (Zhang et al., 2023).
2. RNA samples can be used either for qPCR analysis or RNA sequencing (Figures 2 and 3). Please refer to Figure 1B, C, and D in our publication (Zhang et al., 2023).

Validation of protocol

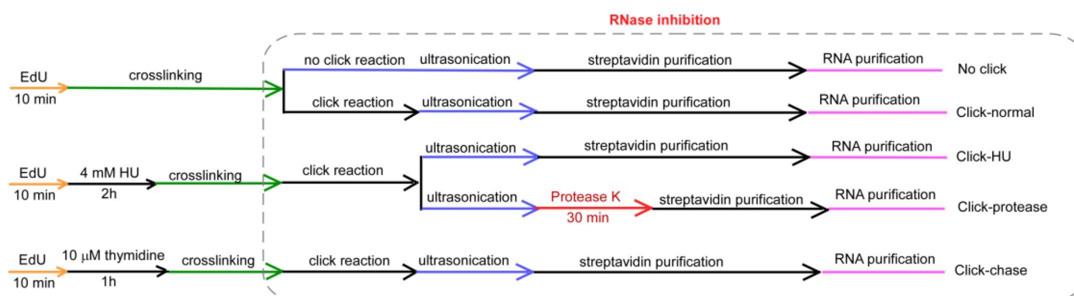


Figure 2. Design of isolate RNAs on nascent DNA (iROND)-based LncRNA screening in mouse embryonic stem cells (mESCs) and NIH3T3 cells (Zhang et al., 2023). We set up the following experimental items: 1) no click group: cells were pulse-labeled by EdU but click reaction was omitted, as a non-specific binding control. 2) Click-normal group: cells were cultured in normal conditions and standard iROND was performed, as a steady-state sample. 3) Click-HU group: cells were pulse-labeled by EdU and treated with 4 mM hydroxyurea (HU) to induce replication stress, as a genotoxic sample. 4) Click-Protease group: all procedures were the same as group 3, with extra treatment of 100 μg/mL protease K for 30 min to degrade protein components. 5) Click-chase group: cells were chase-labeled with thymidine to get a mature chromatin control.

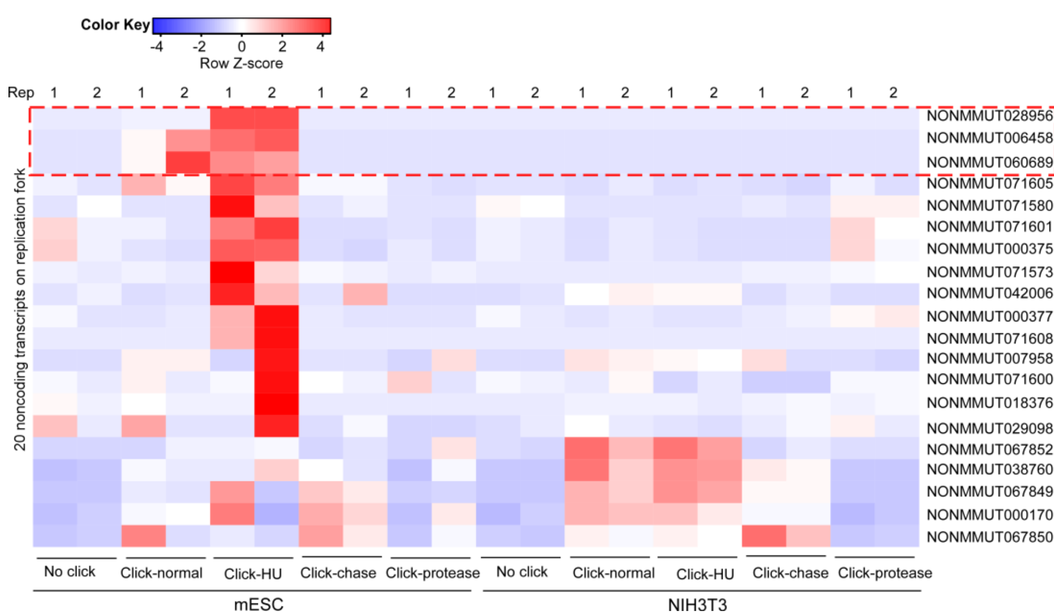


Figure 3. RNA sequencing and bioinformatic comparison of isolate RNAs on nascent DNA (iROND) samples from mouse embryonic stem cells (mESCs) and NIH3T3 cells under indicated conditions (Zhang et al., 2023).

Two biological repeats were performed for each group. Notably, a set of LncRNAs were significantly enriched in mESC Click-HU group.

General notes and troubleshooting

1. Compared with published methods for studying LncRNAs in genomic dynamics or stability, iROND is more specific for purifying the fork-associated LncRNA subpopulation (Gylling et al., 2020; Wang et al., 2021). One limitation is that iROND needs billions of cells for each assay. The more S-phase cells, the easier performing the assay is. In our study, we used mESCs and NIH3T3 cells; both proliferate very fast. This is critical for iROND success. We assume that it might be difficult if low proliferating cell lines are used. Alternatively, synchronization into S phase before EdU labeling may be a reasonable choice.
2. In the steps of EdU labeling and thymidine chase, pre-warming medium is important to minimize disturbance to cells in a very short time.
3. In order to label replication forks as specifically as possible, EdU labeling must be done in 10 min. It is quite hard for only one person to handle many culture dishes in a very short period of time. So, cooperation of two or three people is recommended.

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Competing interests

The authors have no competing interests.

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