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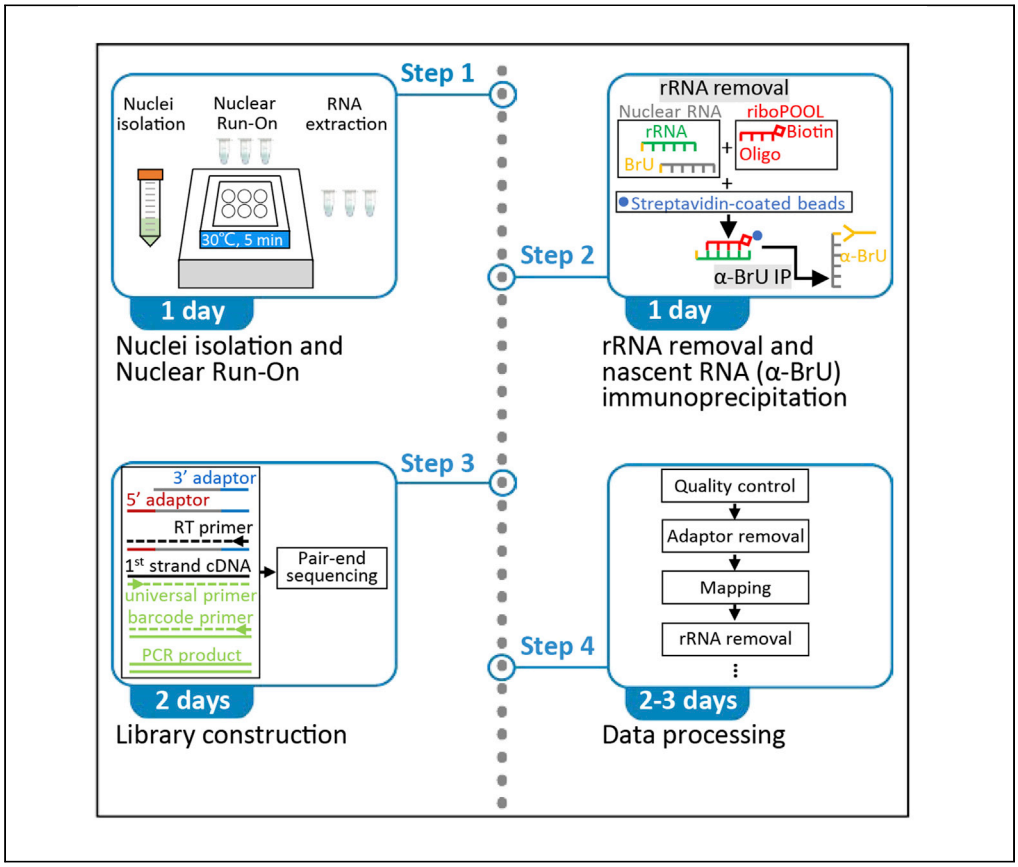
# Protocol for affordable and efficient profiling of nascent RNAs in bread wheat using GRO-seq




Exorbitant sequencing cost is one of the main obstacles limiting the widespread application of Global Run-On sequencing (GRO-seq) to detect transcriptional activity. Here, we describe a more efficient and affordable protocol for GRO-seq that incorporates an rRNA removal step after nuclear RNA isolation and before nascent RNA immunoprecipitation. We have successfully applied this protocol to profile enhancer transcription in allohexaploid bread wheat and increased the proportion of valid data by 20 times.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Highlights

The detailed GRO-seq protocol integrates rRNA depletion

Cost-efficient GRO-seq protocol for detection of eRNA in bread wheat

Applicable for any large complex plant or animal genomes

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# Protocol for affordable and efficient profiling of nascent RNAs in bread wheat using GRO-seq

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## SUMMARY

**Exorbitant sequencing cost is one of the main obstacles limiting the widespread application of Global Run-On sequencing (GRO-seq) to detect transcriptional activity. Here, we describe a more efficient and affordable protocol for GRO-seq that incorporates an rRNA removal step after nuclear RNA isolation and before nascent RNA immunoprecipitation. We have successfully applied this protocol to profile enhancer transcription in allohexaploid bread wheat and increased the proportion of valid data by 20 times.**

**For complete details on the use and execution of this protocol, please refer to Xie et al. (2022).**

## BEFORE YOU BEGIN

GRO-seq was first developed by Core et al. through coupling nuclear run-on assay to high-throughput sequencing in human cells (Core et al., 2008; Gariglio et al., 1974, 1981). Briefly, the ribonucleotide analog [5-bromouridine 5′-triphosphate (BrUTP)] was used during nuclear run-on to label nascent RNA. BrU-incorporated RNA was hydrolyzed, and affinity purified using anti-BrdU antibodies. Finally, BrU-containing RNA fragments were converted to cDNA libraries for sequencing. Thus, the 5′end of GRO-seq reads represents the exact amount, position and orientation of transcriptionally engaged RNA polymerases. In plants, GRO-seq was carried out with fresh tissue from maize and *Arabidopsis* (Erhard et al., 2015; Hetzel et al., 2016; Liu et al., 2018). Afterwards, we published a method for GRO-seq using snap-frozen *Arabidopsis* seedlings (Zhu et al., 2018), which avoided transcriptional perturbations during nuclei isolation from fresh material and demonstrated high reproducibility in multi-sample experiments (Liu et al., 2021).

Recently, we optimized our previous protocol by performing rRNA depletion immediately after nuclear run-on and RNA isolation, which have been proved to be significantly more efficient and affordable during detecting enhancer transcription in bread wheat (Xie et al., 2022). The protocol below provides detailed steps on how to construct rRNA removal GRO-seq libraries using 12-day-old bread wheat seedling. Theoretically, this protocol can be modified for any plant or animal systems with large and/or complex genomes.

*Note:* Make sure that pipette tips and PCR or centrifuge tubes used in this protocol should be nuclease-free.

### Sample collection and storage

Timing: 12 days

The bread wheat (*Triticum aestivum*) cultivar "Chinese Spring" was grown under long-day conditions. Leaf tissue of 12-day-old seedlings was collected by flash freezing with liquid nitrogen, ground into fine powder, and immediately stored at −80°C. Alternatively, tissue leaves can be stored directly at −80°C and ground before isolating nuclei.

### Preparation of stock buffers

Timing: 2 h

Prepare the stock buffers according to key resources table and "stock buffer" in materials and equipment before starting the experiment. Store them at a suitable temperature until use.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| BrdU (IIB5): mouse monoclonal IgG (3:100) | Santa Cruz Biotechnology | sc-32323 |
| Chemicals, peptides, and recombinant proteins | | |
| Phenylmethanesulfonyl fluoride (PMSF) | Sigma-Aldrich | 78830-5G |
| PIPES | Sigma-Aldrich | P8203-50G |
| DTT | Sigma-Aldrich | 43815-25G |
| $MnCl_2$ | Sigma-Aldrich | M3634-100G |
| BSA | Sigma-Aldrich | A1933-25G |
| DEPC | Sigma-Aldrich | D5758-100 mL |
| Hexylene glycol | Sigma-Aldrich | 68340-500 mL |
| $MgCl_2$ (1 M) | Thermo Fisher Scientific | AM9010 |
| KCl (2 M) | Thermo Fisher Scientific | AM9010 |
| NaCl (5 M) | Thermo Fisher Scientific | AM9010 |
| Pepstatin A | ApexBio | A2471-10 mg |
| Aprotinin | ApexBio | A2574-25 mg |
| Percoll | Sigma-Aldrich | P1644-500 mL |
| Triton X-100 | Sigma-Aldrich | X100-100 mL |
| β-mercaptoethanol | Sigma-Aldrich | M6250-100 mL |
| Tris-HCl (1 M, pH 7.0) | Thermo Fisher Scientific | AM9010 |
| Tris-HCl (1 M, pH 8.0) | Thermo Fisher Scientific | AM9010 |
| ATP (10 mM) | Invitrogen | AM8110G |
| GTP (10 mM) | Invitrogen | AM8130G |
| Br-UTP | Sigma-Aldrich | B7166-5 mg |
| CTP (10 mM) | Invitrogen | AM8120G |
| RNase inhibitor | Promega | N2618-10000 U |
| TURBO™ DNase | Thermo Fisher Scientific | 2238G2 |
| antarctic phosphatase | NEB | M0289L |
| T4 Polynucleotide Kinase (T4 PNK) | NEB | M0201L |
| Sarkosyl | Sigma-Aldrich | 61739-5 g |
| $NH_4OAc$ (5 M) | Thermo Fisher Scientific | AM9010 |
| EDTA (500 mM, pH 8.0) | Thermo Fisher Scientific | AM9010 |
| Tween-20 | Sigma-Aldrich | P9416-100 mL |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Polyvinylpyrrolidone (PVP) | Sigma-Aldrich | PVP10-100 g |
| Ammonium persulfate (APS) | Sigma-Aldrich | A3678-25G |
| AMPure XP Beads | Beckman Coulter | A63880 |
| Dynabeads™ M-280 Sheep anti-Mouse IgG | Thermo Fisher Scientific | 11202d |
| Streptavidin Magpoly Beads | Smart-lifesciences | SM01705 |
| Acrylamide/ Bis (19:1) | Thermo Fisher Scientific | AM9022 |
| SYBR® Gold Nucleic Acid Gel Stain | Thermo Fisher Scientific | S11494 |
| Critical commercial assays | | |
| NEXTFLEX® Small RNA-Seq Kit v3 | PerkinElmer | NOVA-5132-06 |
| Pan-Plant riboPOOL | siTOOLs Biotech | PanPlant-10 nmol |
| Micro Bio-Spin™ P-30 Gel Columns, Tris Buffer (RNase-free) | Bio-Rad | 7326250 |
| KAPA HiFi HotStart Real-Time Library Amplification Kit | Kapa Biosystems | KK2702 |
| KAPA HiFi HotStart ReadyMix (2×) | Kapa Biosystems | KK2602 |
| Qubit™ dsDNA HS Assay Kit | Thermo Fisher Scientific | Q32854 |
| Software and algorithms | | |
| Trim Galore 0.6.4 | The Babraham Insititute | https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/ |
| bowtie2 2.3.5.1 | (Langmead and Salzberg, 2012) | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| samtools 1.7 | (Li et al., 2009) | http://www.htslib.org/ |
| bedtools 2.30.0 | (Quinlan and Hall, 2010) | https://bedtools.readthedocs.io/en/latest/ |
| SortMeRNA 2.1b | (Kopylova et al., 2012) | https://github.com/biocore/sortmerna |
| Deposited data | | |
| Traditional and rRNA removal GRO-seq data | GEO | GSE178276 |

## MATERIALS AND EQUIPMENT

| **Stock buffers** | |
|---|---|
| Reagent | Note |
| PMSF (100 mM) | Dissolve 174 mg phenylmethylsulfonyl fluorid (PMSF) in 8 mL isopropanol; fill up to 10 mL and store at −20°C (stable at least for 6 months). |
| PIPES-KOH (200 mM, pH 7.0) | Add 6.05 g PIPES to 80 mL DEPC-$H_2O$; adjust solution to desired pH (7.0) by 10 N KOH; fill up to 100 mL with DEPC-$H_2O$ and store at 4°C (stable at least for 6 months). |
| DTT (1 M) | Dissolve 1.545 g DTT in 8 mL DEPC-$H_2O$; fill up to 10 mL and dispense into 1 mL aliquots; Store at −20°C (stable at least for 6 months). |
| $MnCl_2$ (1 M) | Dissolve 1.979 g $MnCl_2 \cdot 4H_2O$ in 8 mL DEPC-$H_2O$; fill up to 10 mL with DEPC-$H_2O$ and store at 4°C (stable at least for 6 months). |
| BSA (40 mg/mL) | Dissolve 400 mg bovine serum albumin (BSA) in 8 mL DEPC-$H_2O$; fill up to 10 mL with DEPC-$H_2O$ and dispense into 1 mL aliquots; Store at −20°C (stable at least for 6 months). |
| SSPE (20×) | Dissolve 17.53 g NaCl, 2.76 g $NaH_2PO_4 \cdot H_2O$ and 0.74 g EDTA in 80 mL DEPC-$H_2O$; adjust pH to 7.4 by 10 N NaOH; fill up to 100 mL with DEPC-$H_2O$ and store at room temperature (~25°C, stable at least for 6 months). |
| 10% APS (w/v) | Dissolve 1 g ammonium persulphate (APS) in 8 mL DEPC-$H_2O$; fill up to 10 mL and dispense into 1 mL aliquots; Store at −20°C (stable at least for 6 months). |
| DEPC-$H_2O$ | Add 1 mL DEPC (diethylpyrocarbonate) to 1 L dd$H_2O$; shake vigorously and allow the solution to sit overnight (6–8 h) in a fume hood; autoclave the solution to inactivate the DEPC; store at room temperature (~25°C). |

## Work buffers

| Nuclei isolation buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Hexylene glycol (100%) | 2 M | 12.845 mL |
| PIPES-KOH (200 mM, PH 7.0) | 20 mM | 5 mL |
| $MgCl_2$ (1 M) | 10 mM | 500 μL |
| KCl (2 M) | 5 mM | 125 μL |
| β-mercaptoethanol (100%) | 5 mM | 18.33 μL |
| Triton X-100 (20%) | 0.5% | 1.25 mL |
| PMSF (100 mM) | 1 mM | 500 μL |
| Pepstatin A (1 mg/mL) | 2 μg/mL | 100 μL |
| Aprotinin (5 mg/mL) | 2 μg/mL | 20 μL |
| DEPC-$H_2O$ | N/A | 29.65 mL |
| Total | N/A | 50 mL |

| Gradient buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Hexylene glycol (100%) | 0.5 M | 3.211 mL |
| PIPES-KOH (200 mM, PH 7.0) | 5 mM | 1.25 mL |
| $MgCl_2$ (1 M) | 10 mM | 500 μL |
| KCl (2 M) | 5 mM | 125 μL |
| β-mercaptoethanol (100%) | 5 mM | 18.33 μL |
| Triton X-100 (20%) | 0.5% | 1.25 mL |
| DEPC-$H_2O$ | N/A | 43.64 mL |
| Total | N/A | 50 mL |

| 30% Percoll solution | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Hexylene glycol (100%) | 0.5 M | 1.28 mL |
| PIPES-KOH (200 mM, PH 7.0) | 5 mM | 500 μL |
| $MgCl_2$ (1 M) | 10 mM | 200 μL |
| KCl (2 M) | 5 mM | 50 μL |
| β-mercaptoethanol (100%) | 5 mM | 7.33 μL |
| Triton X-100 (20%) | 0.5% | 500 μL |
| Percoll (100%) | 30% | 6 mL |
| DEPC-$H_2O$ | N/A | 11.46 mL |
| Total | N/A | 20 mL |

| 80% Percoll solution | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Hexylene glycol (100%) | 0.5 M | 1.28 mL |
| PIPES-KOH (200 mM, PH 7.0) | 5 mM | 500 μL |
| $MgCl_2$ (1 M) | 10 mM | 200 μL |
| KCl (2 M) | 5 mM | 50 μL |
| β-mercaptoethanol (100%) | 5 mM | 7.33 μL |
| Triton X-100 (20%) | 0.5% | 500 μL |
| Percoll (100%) | 80% | 16 mL |
| DEPC-$H_2O$ | N/A | 1.46 mL |
| Total | N/A | 20 mL |

| 10× Transcription buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl pH 7.2 (1 M) | 250 mM | 250 μL |

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| Reagent | Final concentration | Amount |
|---|---|---|
| $MgCl_2$ (1 M) | 25 mM | 25 μL |
| KCl (2 M) | 25 mM | 12.5 μL |
| $NH_4Cl$ (5 M) | 1 M | 200 μL |
| $MnCl_2$ (1 M) | 5 mM | 5 μL |
| DTT (1 M) | 25 mM | 25 μL |
| DEPC-$H_2O$ | N/A | 482.5 μL |
| Total | N/A | 1 mL |

**Nuclei storage buffer**

| Reagent | Final concentration | Amount |
|---|---|---|
| Tris-HCl pH 7.2 (1 M) | 50 mM | 250 μL |
| $MgCl_2$ (1 M) | 5 mM | 25 μL |
| KCl (2 M) | 5 mM | 12.5 μL |
| $MnCl_2$ (1 M) | 1 mM | 5 μL |
| DTT (1 M) | 5 mM | 25 μL |
| Glycerol (100%) | 50% | 2.5 mL |
| DEPC-$H_2O$ | N/A | 2.1825 mL |
| Total | N/A | 5 mL |

**2× Reaction buffer**

| Reagent | Final concentration | Amount |
|---|---|---|
| Transcription buffer (10×) | 2× | 80 μL |
| ATP (10 mM) | 500 μM | 20 μL |
| GTP (10 mM) | 500 μM | 20 μL |
| Br-UTP (10 mM) | 500 μM | 20 μL |
| CTP (10 μM) | 2 μM | 80 μL |
| RNase inhibitor (2 U/μL) | 40 U/μL | 20 μL |
| Sarkosyl (10%) | 2% | 80 μL |
| DEPC-$H_2O$ | N/A | 80 μL |
| Total | N/A | 400 μL |

**Blocking buffer**

| Reagent | Final concentration | Amount |
|---|---|---|
| SSPE (20×) | 0.5× | 100 μL |
| EDTA (500 mM, pH8.0) | 1 mM | 8 μL |
| Tween-20 (20%) | 0.05% | 10 μL |
| PVP (20%) | 0.1% | 20 μL |
| BSA (40 mg/mL) | 1 mg/mL | 100 μL |
| DEPC-$H_20$ | N/A | 3.762 mL |
| Total | N/A | 4 mL |

**Binding buffer**

| Reagent | Final concentration | Amount |
|---|---|---|
| SSPE (20×) | 0.5× | 100 μL |
| EDTA (500 mM, pH8.0) | 1 mM | 8 μL |
| Tween-20 (20%) | 0.05% | 10 μL |
| DEPC-$H_20$ | N/A | 3.882 mL |
| Total | N/A | 4 mL |

| Low salt buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| SSPE (20×) | 0.2× | 40 μL |
| EDTA (500 mM, pH8.0) | 1 mM | 8 μL |
| Tween-20 (20%) | 0.05% | 10 μL |
| DEPC-$H_20$ | N/A | 3.942 mL |
| Total | N/A | 4 mL |

| High salt buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| SSPE (20×) | 0.5× | 200 μL |
| EDTA (500 mM, pH8.0) | 1 mM | 16 μL |
| Tween-20 (20%) | 0.05% | 20 μL |
| NaCl (5 M) | 150 mM | 240 μL |
| DEPC-$H_20$ | N/A | 7.524 mL |
| Total | N/A | 8 mL |

| TET buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Tris-HCl (1 M, pH 7.4) | 10 mM | 80 μL |
| EDTA (500 mM, pH8.0) | 1 mM | 16 μL |
| Tween-20 (20%) | 0.05% | 20 μL |
| DEPC-$H_20$ | N/A | 7.884 mL |
| Total | N/A | 8 mL |

| Elution buffer | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| DTT (1 M) | 20 mM | 40 μL |
| NaCl (5 M) | 300 mM | 120 μL |
| Tris-HCl (1 M, pH 7.5) | 5 mM | 10 μL |
| EDTA (500 mM, pH8.0) | 1 mM | 4 μL |
| SDS (10%) | 0.1% | 20 μL |
| DEPC-$H_20$ | N/A | 1.806 mL |
| Total | N/A | 2 mL |

*Note:* Work buffers are prepared freshly with DEPC-$H_2O$ and kept on ice; elution buffer should be pre-warmed at room temperature (~25°C); β-mercaptoethanol, DTT and protease inhibitors (PMSF, Pepstatin A and Aprotinin) are added immediately before use.

⚠ CRITICAL: DEPC is a suspected carcinogen and DTT is toxic upon inhalation. Wear gloves and use a fume hood when preparing DEPC-$H_2O$ and DTT stock solution.

## STEP-BY-STEP METHOD DETAILS

### Nuclei isolation and nuclear run-on

Timing: 1 day

Nuclei were isolated by the Percoll gradient procedure as previously described except that the concentration of Triton X-100 was reduced to 0.5% (Zhu et al., 2018). For tissues that have not been tested, pilot experiments are required to optimize the concentration of Triton X-100, which dissolves the plasma membrane and chloroplasts and leaves the nucleus intact. *In vitro* nuclear run-on and nascent RNA purification were performed as previously reported (Zhu et al., 2018).

1. Nuclei isolation.
   a. Prepare nuclei isolation buffer, gradient buffer, 80% Percoll solution, 30% Percoll solution, 10× transcription buffer, 1× transcription buffer and nuclei storage buffer, keep on ice.
   b. Grind leaf tissue with liquid nitrogen to fine powder and dispense into 3 mL aliquots. Add 20 mL cold nuclei isolation buffer to each of three replicates. Mix well and put on ice for 10–15 min. Shake the tube gently every 3 min.
   c. Place 2 layers of nylon mesh (1 layer of 100 μm mesh on the top, 1 layer of 40 μm mesh at the bottom) over the top of a 50 mL centrifuge tube and decant the sample through the mesh. Add nuclei isolation buffer to the filtrate to a final volume of 20 mL.
   d. Prepare a discontinuous Percoll gradient by layering 6 mL 30% Percoll over 6 mL 80% Percoll in a 50 mL tube. Be careful and do not disrupt the 80%–30% interface.
   e. Gently pipette the filtered plant extract onto the top of the 30% Percoll layer. Centrifuge the gradient at 2,000 × *g* for 11 min at 4°C with a swing bucket at a low accelerating and de-accelerating level (±3).
   f. Remove the top layer of nuclei isolation buffer and use a Pasteur pipette to collect nuclei at the 30%–80% interface.
   g. Add cold gradient buffer to the enriched nuclei to a total volume of 20 mL, centrifuge at 2,000 × *g* for 6 min at 4°C.
   h. Gently draw off the supernatant, then resuspend the pellet with 1 mL 1× transcription buffer (pre-cold). Transfer the nuclei to a new 1.5 mL tube, centrifuge at 3,000 × *g* for 3 min at 4°C.
   i. Repeat step h. Then wash nuclei with 1 mL nuclei storage buffer (pre-cold), centrifuge at 5,000 × *g* for 5 min at 4°C.

**Pause point:** Proceed to "Nuclear Run-On" or store nuclei at −80°C. If properly stored, frozen nuclei can be used several weeks after collection.

2. Nuclear Run-On and RNA extraction.
   a. Resuspend nuclei with 100 μL nuclei storage buffer, and add 100 μL 2× reaction buffer, mix well (stir the pellet first with 200 μL tip, then pipette the pellet gently for 30 times). Incubate at 30°C for 5 min immediately. Br-UTP is incorporated into the nascent RNA at this step.
   b. Add 750 μL TRIzol reagent to stop the reaction. Vortex for 60 s and incubate at room temperature (~25°C) for 5–10 min. Add 200 μL chloroform and vortex for 30 s. Spin at 12,000 × *g* for 15 min at 4°C. Transfer the aqueous phase (upper phase) into a new 1.5 mL tube and add 500 μL chloroform, vortex for 30 s.
   c. Spin at 12,000 × *g* for 5 min at 4°C. Transfer the aqueous phase (~560 μL) into two new 1.5 mL tubes (~280 μL each). Add 28 μL (1/10 volume) 5 M $NH_4OAc$, 1 μL glycol-blue (15 μg/μL), 840 μL (3 volumes) 100% EtOH (pre-cold) and mix well by vortexing. Incubate at −80°C for at least two hours or overnight (6–8 h).
   d. Pellet the nuclear RNA from step c by spinning at 12,000 × *g* for 30 min at 4°C.
   e. Discard supernatant and wash the pellet with 1 mL 75% ethanol (pre-cold). Spin at 12,000 × *g* for 5 min at 4°C. Discard the supernatant.
   f. Repeat step e. Then spin briefly and remove the residual 75% EtOH with a 10 μL pipette. Air-dry the pellet for 5–10 min.
   g. Dissolve the RNA in 14 μL DEPC-$H_2O$ and store at −80°C.

⚠ CRITICAL: All the buffers and reaction mixtures should be prepared and kept on ice; As PMSF is toxic, wear gloves while handling.

### Ribosomal RNA (rRNA) removal

Timing: 2–3 h

The rRNA removal step after nuclear RNA isolation and before the affinity purification of nascent RNA is performed according to riboPOOL kit (siTOOLs Biotech) manual with some modifications.

3. Preparation of streptavidin beads.
   a. Resuspend the Streptavidin Magpoly Beads by carefully vortexing at medium speed.
   b. Transfer 100 μL bead suspension per sample to a new 1.5 mL tube. To prepare multiple samples, aliquot bead suspension for 3 samples (i.e., 300 μL) to a single tube.
   c. Place tube on magnetic rack, aspirate supernatant.
   d. Add 100 μL depletion buffer per sample (i.e., 300 μL for 3 samples) and resuspend the beads.
   e. Place on magnetic rack, aspirate supernatant.
   f. Repeat step d and e.
   g. Resuspend beads in 40 μL depletion buffer per sample (i.e., 120 μL for 3 samples). Leave the beads at room temperature (~25°C) until use.
4. Hybridization of riboPOOL to RNA.
   a. To the 14 μL RNA sample from step 2g add and mix 1 μL riboPOOL and 5 μL hybridization buffer.
   b. Incubate at 68°C for 10 min to denature RNA.
   c. Allow to cool slowly from 68°C to 37°C (3°C/min) for optimal hybridization.
5. rRNA depletion.
   a. Briefly centrifuge the tube containing ~20 μL hybridized riboPOOL and nuclear RNA (from step 4c).
   b. Dispense 40 μL beads (from step 3) to the hybridized riboPOOL/RNA solution and mix well by pipette.
   c. Incubate at 37°C for 15 min, follow by a 50°C incubation for 5 min. Pipette the mixture every 5 min.
   d. Briefly spin down droplets and place the tube on magnetic rack. Very carefully transfer the supernatant to a new 1.5 mL tube.
   e. Beads clean-up: add 90 μL (1.8×) AMPure Beads to the supernatant and mix well by pipette; immediately add 270 μL isopropanol and mix well by pipette; incubate for 5 min at room temperature (~25°C) and magnetize sample for 5 min or until solution appears clear; discard supernatant and wash the beads by 180 μL 80% EtOH twice; spin briefly and remove the residual 80% EtOH. Air dry the beads for 3–5 min. Remove the tube from the magnetic rack and resuspend the beads in 20 μL DEPC-$H_2O$ by pipetting volume up and down. Incubate the bead suspension for 3 min at room temperature (~25°C). Magnetize sample for 3 min and transfer 20 μL supernatant to a new tube.

**Pause point:** Proceed to "RNA immunoprecipitation" or store RNA at −80°C (stable for up to a month).

⚠ CRITICAL: Always use freshly prepared 80% ethanol and do not incubate the beads with 80% ethanol for more than 30 s.

## RNA immunoprecipitation

**Timing: 6–8 h**

The rRNA depleted nuclear RNA is subject to RNA fragmentation, DNA removal and de-phosphorylation sequentially. Then, Br-UTP containing nascent RNA is affinity purified using anti-BrdU antibodies.

6. Anti-BrdU antibody conjugation to magnetic beads.
   a. Prepare binding buffer.

b. Resuspend the Dynabeads™ M-280 Sheep Anti-Mouse IgG by carefully vortexing at medium speed.
c. Transfer 50 μL bead suspension per sample to a new 1.5 mL tube.
d. Place tube on magnetic rack, aspirate supernatant.
e. Resuspend the beads in 1 mL binding buffer and add 30 μL anti-BrdU antibody (IIB5) per sample. Rotate at 4°C for 4–6 h. (During this step, proceed to steps 7 and 8).

7. RNA fragmentation and purification.
   a. The rRNA depleted nuclear RNA from step 5e (20 μL) is subjected to base hydrolysis by adding 5 μL 1 M NaOH (freshly prepared) and incubate on ice for 40 min. Then add 25 μL 1 M Tris-HCl (pH 7.0) to neutralize the reaction.
   b. RNA is desalted through a p-30 RNase-free spin column, according to the manufacturer's instructions (https://www.bio-rad.com/sites/default/files/webroot/web/pdf/lsr/literature/10000069982.pdf).
8. DNA removal and de-phosphorylation.
   a. Adjust the eluted RNA volume to 50 μL with DEPC-$H_2O$. Then add 6.7 μL DNase buffer and 3.5 μL RNase-free DNase (2 U/mL, 2238G2), mix by vortexing. Briefly spin down droplets and incubate at 37°C for 30 min.
   b. RNA is purified again through a p-30 column.
   c. Adjust the eluted RNA volume to 70 μL. Then add 8.5 μL 10× antarctic phosphatase buffer, 1 μL RNase Inhibitor (Promega, N2615) and 5 μL antarctic phosphatase to the fragmented RNA and incubate for 1 h at 37°C. (During this step, proceed to step 9).
9. Immunoprecipitation.
   a. Prepare binding buffer, blocking buffer, low salt buffer, high salt buffer, and TET buffer, keep on ice.
   b. Put the tube containing antibody conjugated beads (from step 6e) on magnetic rack and aspirate the supernatant. Block the beads in 1 mL blocking buffer for 1 h at 4°C with rotation.
   c. Add 500 μL binding buffer to the sample (from step 8c) and denature the RNA at 65°C for 5 min, then immediately put on ice.
   d. Transfer the denatured RNA to beads in 500 μL binding buffer and allow to bind for 1 h at 4°C with rotation.
   e. After binding, spin at 1,500 × *g* for 5 min at 4°C, remove supernatant. Then beads are washed once in 1 mL low salt buffer, twice in high salt buffer and twice in TET buffer at 4°C with 5 min rotation.
   f. Elute the BrU-incorporated RNA in 250 μL elution buffer at room temperature (~25°C) with 10 min rotation.
   g. Add 800 μL TRIzol reagent to stop elution. Vortex for 1 min and incubate at room temperature (~25°C) for 5–10 min. Add 200 μL chloroform and vortex for 30 s. Spin at 12,000 × *g* for 15 min at 4°C. Transfer the aqueous phase (upper phase) into a new 1.5 mL tube and add 500 μL chloroform, vortex for 30 s.
   h. Ethanol precipitation of the eluted RNA refers to step 2c.

## Library construction

**Timing: 2 days**

The enriched BrU-incorporated RNA is end-repaired by PNK treatment and then subject to small RNA library construction. Then the target cDNA library is recovered by PAGE size selection (170 bp-330 bp) and quantified by Qubit.

10. PNK treatment.
    a. Pellet and wash the RNA (from step 9h) as described in steps 2d–f.
    b. Dissolve the RNA in 45 μL DEPC-$H_2O$. Denature the RNA at 65°C for 5 min and immediately cool on ice for 2 min.

c. Add 5.2 μL T4 PNK buffer, 1 μL RNase inhibitor and 1 μL T4 PNK to the denatured RNA. Incubate at 37°C for 1 h.
d. Add 300 μL TRIzol reagent to stop elution. Vortex for 1 min and incubate at room temperature (~25°C) for 5–10 min. Add 100 μL chloroform and vortex for 30 s. Spin at 12,000 × *g* for 15 min at 4°C. Transfer the aqueous phase (upper phase) into a new 1.5 mL tube and add 200 μL chloroform, vortex for 30 s.
e. Ethanol precipitation of RNA refers to step 2c.

11. Library construction.
a. Pellet and wash the RNA (from step 10e) as described in steps 2d–f.
b. Dissolve the RNA in 10.5 μL DEPC-$H_2O$ and proceed to small library construction using NEXTflex™ Small RNA-Seq Kit v3 according to the manufacturer's manual exactly except for the PCR amplification (https://perkinelmer-appliedgenomics.com/wp-content/uploads/marketing/NEXTFLEX/small_rna/5132-05-NEXTflex-Small-RNA-Seq-v3_19.01.pdf):

| **PCR reaction mixture:** | |
|---|---|
| Reagent | Amount |
| Purified first strand synthesis product | 18 μL |
| 2× KAPA HiFi HS ReadyMix | 20 μL |
| NEXTflex™ Universal Primer | 1 μL |
| NEXTflex™ Barcoded Primer (different barcoded primers should be used for each sample that will be multiplexed for sequencing) | 1 μL |
| Total | 40 μL |

| **PCR cycle:** | | | |
|---|---|---|---|
| Steps | Temperature | Time | Cycles |
| Initial Denaturation | 95°C | 3 min | 1 |
| Denaturation | 98°C | 20 s | 5+N cycles |
| Annealing | 60°C | 30 s | |
| Extension | 72°C | 40 s | |
| Final extension | 72°C | 2 min | 1 |
| Hold | 4°C | forever | |

*Note:* Run for 5 cycles first, then take out 5 μL sample (keep the remaining 35 μL PCR sample mix on ice) and use KAPA HiFi Real-time PCR Library Amplification Kit to determinate the remaining PCR N cycle number to generate optimal library for sequencing. Add 5 μL 2× KAPA PCR mix to the 5 μL sample, mixed well. Take out 10 μL standard control 1–4, two repeats each. Choose the N cycles which make sure the sample fluorescence is between standard control 1 and standard control 3 fluorescence. Lastly amplify the remaining 35 μL PCR sample mix for additional N cycles (normally no more than 10 cycles). It is not recommended to over amplify the sample.

12. Gel recovery.
a. To make an 8% polyacrylamide TBE gel (non-denaturing), mix the follow items in a 15 mL corning tube: 2.2 mL 5× TBE, 2.2 mL 40% polyacrylamide stock solution, fill up to 11 mL with dd$H_2O$, mix well; then add 66 μL 10% APS, 5.1 μL TEMED, mix well. Put the mixture into a 1.5 mm thick glass cassette (Bio-Rad protein system IV) with a 10-well comb. Wait for half an hour.
b. Add 6 μL of 6× loading buffer (provided by NEXTflex™ Small RNA-Seq Kit v3) to the PCR product (from step 11b).
c. Resolve the PCR product on the gel, then electrophoresis at 130 V for 1–2 h in 1× TBE electrophoresis solution till the Xylene Cyanol reach the middle of gel and the Bromophenol Blue go off the gel. Stain the gel with SYBR® Gold Nucleic Acid Gel Stain /TBE (1/10,000) for 5–15 min.

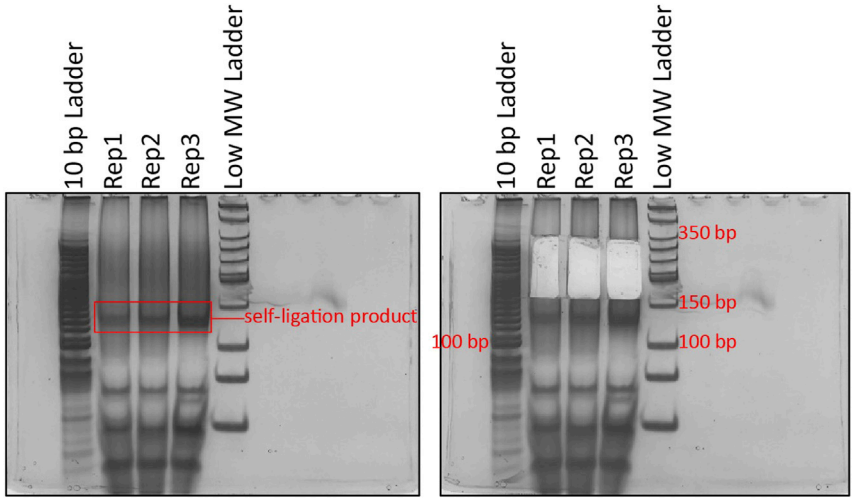


**Figure 1. rRNA removal GRO-seq cDNA resolved on a PAGE**

Left, a typical PAGE of rRNA removal GRO-seq cDNA library. Right, the PAGE after gel slicing. Molecular weight and adapter self-ligation product are indicated.

d. Take picture of the gel and excise the gel slices between 170 bp and 330 bp (Figure 1). Recover the cDNA by soaking the grinded gel (use a fire sealed 1 mL tip) in 400 μL DNA gel elution buffer (300 mM NaCl and 1 mM EDTA) overnight (6–8 h) at 30°C on thermo rotator (1,200× rpm).

e. Pass the gel through a 0.45 μm spin column by centrifugation at 8,000 × *g* for 5 min (Corning, V123041), then precipitate the cDNA by adding: 1 μL glycol-blue (15 μg/μL), 35 μL (1/10 volume) 3 M NaOAc, 1,050 μL (3 volumes) 100% EtOH. Incubate at −80°C for at least two hours or overnight (6–8 h).

f. Pellet, wash and resuspend the cDNA as described in steps 2d–g.

⚠ CRITICAL: Resolving the PCR product on a TBE native PAGE can serve as a check point for library preparation. Optionally, to save time and simplify operations, experienced experimenters can use magnetic beads for size selection instead of gel recovery if there is no obvious adapter self-ligation.

13. Quality check of the cDNA library.

a. Quantify the library by Qubit dsDNA HS Assay Kit according to the manufacturer's manual (https://www.thermofisher.cn/document-connect/document-connect.html?url=https://assets.thermofisher.cn/TFS-Assets%2FLSG%2Fmanuals%2FQubit_dsDNA_HS_Assay_UG.pdf) and check the size distribution with a Fragment Analyzer.

## EXPECTED OUTCOMES

This protocol describes the construction of GRO-seq libraries that incorporates the rRNA depletion step. The final cDNA libraries can be applied to next-generation sequencing (NGS) to generate nascent transcriptome datasets. For more details, please refer to the recent publication (Xie et al., 2022).

The library preparation with 1 μg of input nuclear RNA yields approximately ~150 ng cDNA for each library with a size distribution between 157 bp and 326 bp (average size 208.9 bp) (Figure 2).

In total, ~2,587 M and 994 M raw reads were obtained for the traditional and rRNA removal GRO-seq libraries, respectively (Table 1). After cleaning and removal of reads originating from chloroplasts, mitochondria and rRNA, only ~20 M reads were uniquely mapped to the International Wheat

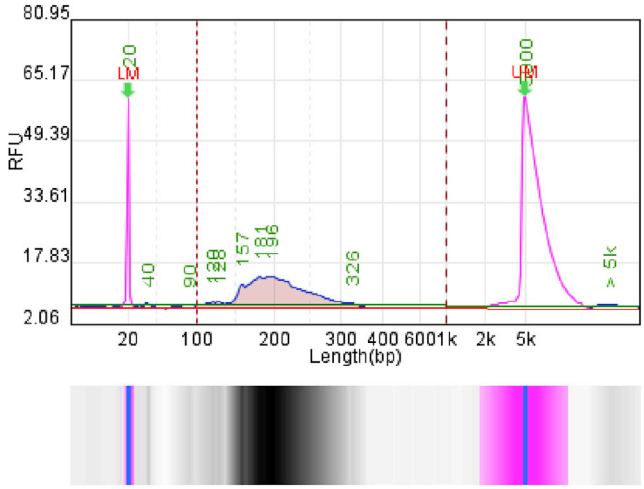


**Figure 2. The size distribution of rRNA removal GRO-seq cDNA were analyzed on a 2100 bioanalyzer instrument (Agilent)**

LM (lower marker) and UM (upper marker) are indicated.

Genome Sequencing Consortium (IWGSC) reference sequence (version 1.0) for the traditional GRO-seq libraries (average mapping ratio 0.82%), compared to 158 M uniquely mapped reads for rRNA removal GRO-seq libraries (average mapping ratio 16.67%). The uniquely mapping ratio is sharply increased by more than 20 times by performing the rRNA removal step. In addition, correlations of the biological replicates were also greatly improved after rRNA removal (Table 2).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 2–3 days

The scripts for data cleaning, genomic mapping and rRNA removal are described below. Further analysis depends on the specific study purpose. All following steps are applied to each GRO-seq library referred hereafter as <${sample}>.

1. Remove low-quality reads and trim adapters using Trim Galore.

```
> trim_galore -q 25 –phred 33 –paired ${sample}.R1.fq.gz ${sample}.R2.fq.gz -o ${sample}
```

2. Reads mapping and filtering multiple alignments.
   a. Align reads to an indexed reference wheat genome <reference_index>.

```
> bowtie2 -x reference_index -1 ${sample}_val_1.fq.gz -2 ${sample}_val_2.fq.gz | samtools
view -Sb -h - |samtools sort -o ${sample}.bam
```

   b. Filter unmapped reads and low quality alignments (q=0).

```
> samtools view -b -h -F 4 -q 1 -f 2 ${sample}.bam -o ${sample}.filter.bam
```

   c. Retrieve uniquely mapped reads.

```
> samtools view -h -f 64 ${sample}.filter.bam |grep -v "XS:i" > ${sample}.unique.bam
```

d. Retrieve alignments with the highest score for multiple alignments.

```
> samtools view ${sample}.filter.bam | cut -f 1,12,13 | paste - - | awk '$1==$4' | grep "XS:i" |
sed 's/AS:i:/AS:i:\t/g' | sed 's/XS:i:/XS:i:\t/g' | awk '$3!=$5||$8!=$10' | cut -f 1 > best.id

> samtools view -f 64 ${sample}.filter.bam | grep -F -w -f best.id - > ${sample}.best.bam
```

e. Merge the files from 2c and 2d.

```
> samtools merge ${sample}.rmmul.bam ${sample}.unique.bam ${sample}.best.bam
```

3. Remove reads from rRNA and plastids using sortmerna (If the raw sequencing data is not very large, rRNA-derived reads can be removed at the beginning).
   a. Covert bam file to fastq file.

```
> samtools sort -n ${sample}.rmmul.bam -o ${sample}.sort.bam

> bedtools bamtofastq -i ${sample}.sort.bam -fq ${sample}.mapped.R1.fq -fq2 ${sample}.
mapped.R2.fq
```

b. Filter reads mapped to rRNA and plastid genome by SortMeRNA.

```
> sortmerna –ref rRNA_database;plastid_database –reads ${sample}.mapped.R1.fq –num_align-
ments 1--fastx –aligned ${sample}.mapped_rRNA –other ${sample}.mapped_non_rRNA
```

c. Retrieve non-rRNA/plastid alignments.

```
> bamToBed -i ${sample}.rmmul.bam > ${sample}.rmmul.bed

> cat ${sample}.mapped_non_rRNA.fq|paste - - - -|cut -d ' ' -f 1 |sort -k1,1 | sed 's/^@//' |sort
-k1,1 |grep -F -w -f - ${sample}.rmmul.bed > ${sample}.mapped.bed
```

4. Remove alignments located in rRNA gene locates.

```
> bedtools subtract -a ${sample}.mapped.bed -b wheat_rRNA.bed > ${sample}.bed
```

## LIMITATIONS

The current rRNA removal GRO-seq protocol has efficiently decreased the sequencing cost as well as the operational time and complexity, whereas a further cut of the number and duration of steps is still in need, and the rRNA removal also has room to improve. Moreover, due to the limitation of labeling efficiency, GRO-seq can only be performed at the level of $10^6$ cells, and the protocol for less starting material needs to be further developed.

## TROUBLESHOOTING

### Problem 1

Large amount of starting material and low yield of nuclear RNA.

**Table 1. Mapping profiles of traditional and rRNA removal GRO-seq libraries**

| Sample | Total reads | Clean reads | Uniquely mapped reads | Mapping ratio |
|---|---|---|---|---|
| Traditional_rep1 | 1,139,417,843 | 869,763,902 | 14,277,497 | 1.25% |
| Traditional_rep2 | 1,447,268,297 | 1,316,343,990 | 5,658,647 | 0.39% |
| rRNA removal_rep1 | 245,854,120 | 224,902,665 | 43,964,944 | 17.88% |
| rRNA removal_rep2 | 401,883,051 | 375,358,038 | 68,476,886 | 17.04% |
| rRNA removal_rep3 | 345,639,801 | 311,838,932 | 52,185,704 | 15.10% |

### Potential solution

This problem may be caused by the low yield of nuclei isolation. The detergent concentration in the nuclei isolation buffer and the Percoll concentration in the gradient buffer are critical and may require further testing. And nuclei isolation checkpoints can be set to examine nuclear integrity and yield under the microscope.

### Problem 2

Adapter self-ligation product contamination.

### Potential solution

This problem may be caused by an excess of adapters relative to the run-on RNA in the RNA ligation reaction. Yields of run-on RNA after anti-BrU affinity purification typically range from 1-10 ng, which can be quantified using Qubit Fluorometer. If the run-on RNA was lower than 1 ng, a 5- to 10-fold dilution of the adapters is recommended. Size selection via polyacrylamide gel between the 3′- and 5′- adapter ligation should also reduce adapter self-ligation.

### Problem 3

Highly abundant reads derived from rRNA are found in the GRO-seq results.

### Potential solution

The commercially available rRNA removal kits are based on biotin labeled probes complementary to rRNAs, which have been tested by the manufacturer for a few species. Therefore, it is ideal to perform a pilot experiment on species that will undergo GRO-seq. Furthermore, after rRNA removal, approximately 10% of nuclear RNA was retained, which can be used as a checkpoint. If the recovery exceeds 15%, a second round of rRNA removal is recommended.

### Problem 4

Low yields of nascent RNA.

### Potential solution

Immunoprecipitation of BrU labeled RNA is a reliable purification method. In a test using synthesized spike-in RNAs, approximately 88% of the BrU labeled RNA and 0.4% of non-BrU labeled RNA was recovered using anti-BrU beads, indicating a high yields and low background protocol [7]. In any case, if the nascent RNA yields are low, the issue of anti-BrU immunoprecipitation can be ruled out first with a synthesized spike-in RNA pull-down test. It is then suggested to check whether the quality or quantity of the nucleus is insufficient, or other impurities affect the run-on efficiency,

**Table 2. Pearson correlations of the biological replicates**

| Sample | Traditional_rep1 | Traditional_rep2 | rRNA_removal_rep1 | rRNA_removal_rep2 | rRNA_removal_rep3 |
|---|---|---|---|---|---|
| Traditional_rep1 | 1.000 | 0.461 | 0.429 | 0.467 | 0.446 |
| Traditional_rep2 | 0.461 | 1.000 | 0.139 | 0.159 | 0.149 |
| rRNA_removal_rep1 | 0.429 | 0.139 | 1.000 | 0.988 | 0.996 |
| rRNA_removal_rep2 | 0.467 | 0.159 | 0.988 | 1.000 | 0.996 |
| rRNA_removal_rep3 | 0.446 | 0.149 | 0.996 | 0.996 | 1.000 |

resulting in only a small amount of RNA being labeled by BrU. This possibility can be diagnosed by quantitative reverse transcription PCR of internal reference genes, such as *Actin*, after anti-BrU immunoprecipitation.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhicheng Dong (zc_dong@gzhu.edu.cn).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

For complete details on data analysis generated using rRNA removal GRO-seq, please refer to Genbank: GSE178276 (Xie et al., 2022) and quantification and statistical analysis section.

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## AUTHOR CONTRIBUTIONS

Z.D. conceived and designed the experiment. J.Z. and M.L. established the original GRO-seq protocol and the pipeline for data analysis and quality assessment. Y.C. optimized the protocol by integrating the rRNA removal step. Y.X. analyzed the original and rRNA removal GRO-seq data under the supervision of Y.Z. Z.L. performed validation experiments. Y.C., J.Z., M.L., and Z.D. wrote the protocol.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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