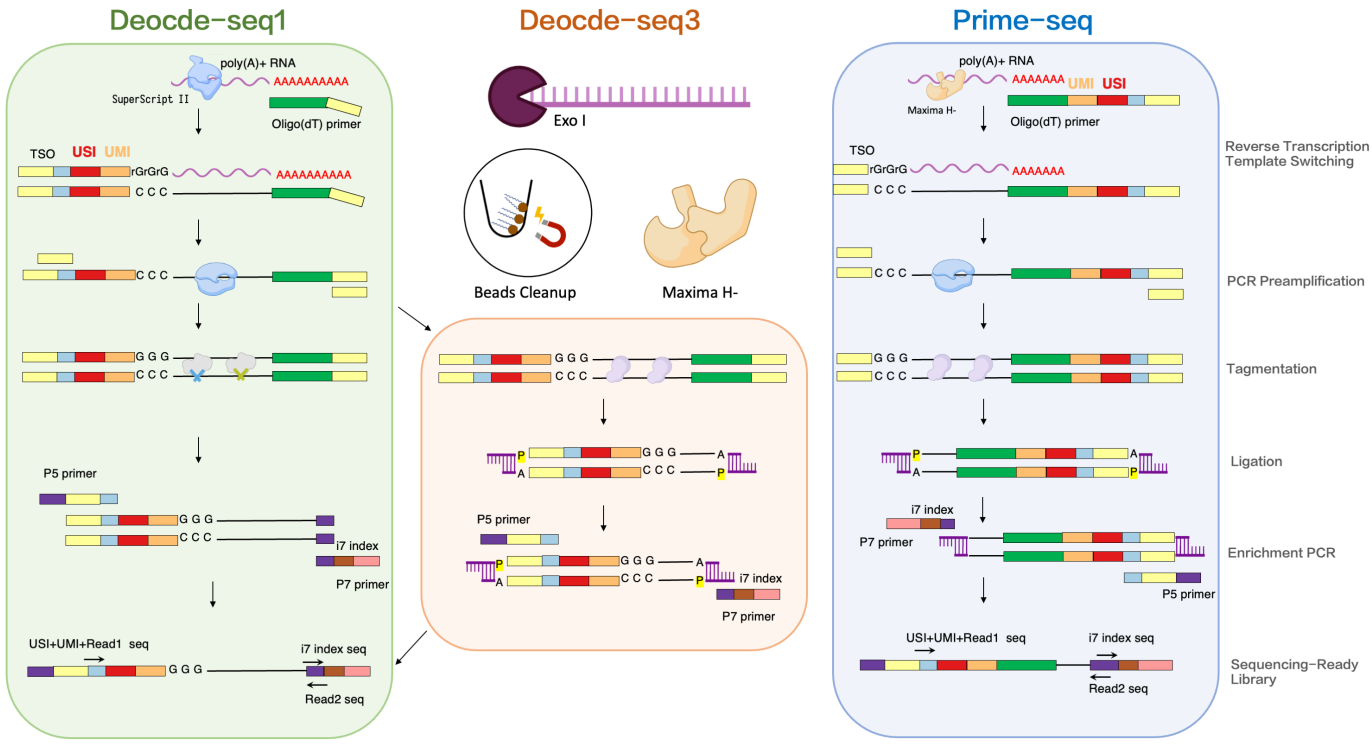
# Decode-seq3

TuLab IGDB CAS, 2024-01

# Overview

- **Decode-seq1 protocol**: http://tulab.genetics.ac.cn/media/filer_public/76/35/763595a7-0ea6-46ed-9220-9e38717ec384/decode-seq_protocol.pdf
- **prime-seq protocol**: https://www.protocols.io/view/prime-seq-81wgb1pw3vpk/v2
- Decode-seq3 是在 Decode-seq1 的基础上做出适当修改，主要参考了 prime-seq 在 RNA 提取、反转录酶使用、建库等方面的改进。三种方法的主要区别如下图所示:




- Decode-seq3 文库构建流程如下图所示：


# Preparation

## 试剂购买及配制

### 试剂购买

| name | cat |
| --- | --- |
| Sera-Mag Speed Beads | Ge Healthcare cat. 65152105050250 |

| name | cat |
| --- | --- |
| PEG 8000, Poly(ethylene glycol) | Sigma Aldrich cat. 89510 |
| EDTA (0.5M, PH8.0) | BioRoYee cat. N-R1007, |
| Igepal(10%) 非离子型表面活性剂 | Sigma Aldrich cat. I8896 |
| TE Buffer, PH8.0 | Life Technologies cat. AM9849 |
| NaCl (5 M), 无 RNase | Invitrogen cat. AM9760G |
| Tris-HCl (1M, pH 8.0) | BioRoYee cat. NR0073 |
| Maxima H Minus Reverse Transcriptase | Thermo Scientific cat. EP0753 |
| dNTPs (10 mM) | Thermo Scientific cat. R0192 |
| Exonuclease I (E.coli) | NEB cat.M0293V |
| KAPA HiFi 2x ReadyMix | KAPA cat. kk2602 |
| NEBNext Ultra II FS DNA Library Prep Kit for Illumina | NEB cat. E7805S |
| Buffer EB | Qiagen cat. 19086 |
| SPRI select beads | Beckman Coulter, cat. B23317 |

- 📓 Ref: Decode-seq1 protocol 和 prime-seq protocol (见 Overview 部分)

**试剂配制**

- **PEG Solution** (22%)
  - Prepare PEG Solution (22%) by adding all ingredients to a 50 mL falcon tube. Do not add the total amount of water until after PEG is completely solubilized.
  - Incubate at 40 °C and vortex regularly until PEG is completely dissolved
  - 📓 Notes: 最终 49ml PEG Solution 中加入了1ml beads，相当于beads浓度为 2%，应该足够吸附DNA。

| Reagent | Amount |
| --- | --- |
| PEG 8000 | 11 g |
| NaCl (5M) | 10 mL |
| Tris-HCl (1M, pH 8.0) | 500 µL |

| Reagent | Amount |
|---|---|
| EDTA (0.5M) | 100 µL |
| IGEPAL (10% solution) | 50µL |
| UltraPure Water | up to 49 mL |
| Total | 49mL |

- **Cleanup Beads** (in 22% PEG Solution)
  i. Resuspend **Sera-Mag Speed Beads** carefully and pipette **1 ml** of bead suspension into a 1.5 mL tube
  ii. Place on magnet stand and remove storage buffer
  iii. Add **1 ml** of TE Buffer and resuspend beads
  iv. Place on magnet stand and remove supernatant
  v. Repeat wash step one more time
  vi. Add 900 µl TE Buffer and resuspend beads
  vii. Add the washed Sera-Mag Speed Beads to the 49mL PEG Solution (22%) and mix well
- **Annealing buffer** (10mM Tris pH8, 50mM NaCl, 1 mM EDTA)
  - 990µL TE + 10µL 5M NaCl

# Primer sequence

- primers: thaw on ice

| Primer name | Primer sequence |
|---|---|
| 5bc-RTb | Biotin-ACACTCTTTCCCTACACGACGC AGTCG T30 VN |
| 5bc-TSO | ACACTCTTTCCCTACACGACGCTCTTCCGATCT BC6 N17 /rG/rG/rG/ |
| single-Pb(同pm-Preamp) | Biotin-ACACTCTTTCCCTACACGACGC |

| Primer name | Primer sequence |
| --- | --- |
| pm-Adapter-Se | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGT |
| pm-Adapter-Anse | /5Phos/CTGTCTCTTATACACATCT |
| prime-seq Adapter | pm-Adapter-Se 和 pm-Adapter-Anse 的退火产物 |
| P5R1(同 pm-3enrich) | AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT |
| i7 index primer | CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG |

- 退火步骤：
  - 用 Annealing buffer 稀释引物（pm-Adapter-Se 和 pm-Adapter-Anse）至 100μM，分别取 30μL，等量混合；
  - 在PCR仪上运行退火程序；

| | | Cycles | Temperature | Time |
| --- | --- | --- | --- | --- |
| Advanced Protocol (example in which the oligonucleotide pair has a $T_m$ of 55°C)* | Step 1: | 1 | 95°C | 5 min |
| | Step 2: | 40* | 95°C (-1°C/cycle) | 1 min |
| | Step 3: | 1 | 55°C | 30 min |
| | Step 4: | 20* | 55°C (-1°C/cycle) | 1 min |
| | Step 5: | | 4°C | HOLD |

*The number of cycles in Step 2 and 4 depends on the $T_m$ of the oligonucleotides to be annealed.

  - 退火产物 prime-seq Adapter 此时浓度为 50μM，稀释至 1.5μM 后使用。

# RNA extraction

- RNA 提取可自行选择方法(Trizol、Qiagen柱子、beads）
- Beads 提取 RNA 主要包括下列步骤(参考 prime-seq protocol)：
  - Sample Collection
  - Proteinase K Digest

- Bead Clean Up
- DNase I Digest

# Reverse Transcription (Decode3)

- Prepare RT Mix:

| Reagent | Well | Plate |
|---|---|---|
| Maxima H Minus RT | 0.15 µL | 16.5 µL |
| Maxima RT Buffer (5x) | 2 µL | 220 µL |
| dNTPs (10 mM) | 1 µL | 44*2.5 µL |
| 5bc-RTb primer(10uM) | 1 µL | |
| Total | 4.15 µL | |

- add RNA samples per well (4-40ng RNA), final volumn 5.75 µL using RF water.
- Add 0.1 µL **5bc-TSO** (100 µM) per well.
  - 实验室有 30 种 5bc-TSO，其中 5bc-TSO30，5bc-TSO35，5bc-TSO38 测试效果不好，分别用 5bc-TSO5，5bc-TSO6，5bc-TSO7 代替。

```
5bc-TSO11, 5bc-TSO12, 5bc-TSO13, 5bc-TSO14, 5bc-TSO15,
5bc-TSO16, 5bc-TSO17, 5bc-TSO18, 5bc-TSO19, 5bc-TSO20,
5bc-TSO21, 5bc-TSO22, 5bc-TSO23, 5bc-TSO24, 5bc-TSO25,
5bc-TSO26, 5bc-TSO27, 5bc-TSO28, 5bc-TSO29, 5bc-TSO30(TSO5),
5bc-TSO31, 5bc-TSO32, 5bc-TSO33, 5bc-TSO34, 5bc-TSO35(TSO6),
5bc-TSO36, 5bc-TSO37, 5bc-TSO38(TSO7), 5bc-TSO39, 5bc-TSO40
```

- Incubate for **1:30** at **42°C**

# cDNA pooling & beads purification (Decode3/pm)

- Pool all wells cDNA into a 1.5/2 mL tube
- Add **10 µl** of Cleanup Beads (22% PEG) for **each sample** for a **1:1** ratio

    - 📓 Notes: The EDTA in the Cleanup Beads will inactivate the RT)
    - 📓 Notes: Final PEG 11%, 1.8x Ampure beads 为 PEG 18%*1.8/2.8 = 11.57%，left side size 约为 150bp(参考 beads 测试电泳图)
- Incubate for 5min at RT to allow binding of the cDNA onto beads
- Place the tube on the magnet stand until clear (~3 min) and discard supernatant
- Wash with **1 mL** of **80% EtOH** while the tube is on the magnet. Discard the supernatant.
    - 📓 Notes: Volume of EtOH should be adjusted depending on the number of samples. More samples will require more EtOH to cover the beads completely.
    - ❗ When washing the sample with 80% ethanol, keep the sample tube on the magnetic frame and do not stir the magnetic beads.
    - ❗ The volume of 80% ethanol should be larger than the original RNA and beads mix
- Repeat wash step once more
- Air dry beads for **5min** (regularly check the beads and avoid over-drying)
- Elute the beads in **17 µL** of UltraPure Water
- Incubate for **5min** at RT and transfer to a new PCR tube or plate.

# Exonuclease I Treatment (Decode3/pm)

- Add **2 µl** of **ExoI Buffer (10x)** and **1 µl** of **Exonuclease I**. Incubate as follows:

| Step | Temperature | Time |
|---|---|---|
| Incubation | 37°C | 20 min |
| Heat Inactivation | 80°C | 10 min |
| Storage | 4°C | hold |

- Mix each sample (20 µL per well) with **16 µl** of Cleanup Beads (22% PEG) for a **1:0.8** ration
    - 📓 Notes: final PEG 9.778%, 1.2x anpure beads 为 PEG 18%*1.2/2.2 = 9.82%，left side size 约为 200-250bp
- Incubate for 5min at Room temperature to allow binding of the cDNA onto beads
- Place the tube on the magnet stand until clear (~3 min) and discard supernatant
- Wash with 50 µl of 80% EtOH while the tube is on the magnet. Discard the supernatant
- Repeat wash step once more
- Air dry beads for 5min
- Elute the beads in **20 µl** of UltraPure Water
- Incubate for 5min at RT and transfer to a new PCR tube or plate

# Full length cDNA Amplification (Decode3/pm)

- Prepare Pre Amplification Mix

| Reagent | 1x |
|---|---|
| KAPA HiFi 2x | 25 µL |
| single-Pb (10uM) | 3 µL |
| NF Water | 2 µL |
| Total | 30 µL |

- Add **30 µl** Pre Amplification Mix to sample
- Incubate the Pre Amplification PCR as follows:

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Initial Denaturation | 98°C | 3 min | 1 cycle |
| Denaturation | 98°C | 15 sec | 10 cycles* |
| Annealing | 65°C | 30 sec | |
| Elongation | 72°C | 4 min | |
| Final Elongation | 72°C | 10 min | 1 cycle |
| Storage | 4°C | hold | |

- Adjust the number of cycles based on input (sample number, cell number, or concentration). As a general guide we recommend:

| Total RNA Input | Cycles |
|---|---|
| 10 ng | 16 |
| 50 ng | 14 |
| 100 ng | 12 |
| 500 ng | 10 |

| Total RNA Input | Cycles |
|---|---|
| 1000 ng | 9 |

# cDNA Bead Purification (Decode3/pm)

- Mix sample with **40µl** Cleanup Beads (22% PEG) for a ratio of 1:0.8
- Incubate for 5min at 20°C (Room Temp)
- Place the tube on the magnet stand until clear (~3 min) and discard supernatant
- Wash with 100 µl of 80% EtOH while the tube is on the magnet. Discard the supernatant
- Repeat wash step once more
- Air dry beads for 5min
- Elute cDNA in 10 µl UltraPure Water
- Incubate for 5min at RT and transfer to a new PCR tube or plate

🛑 Stopping Point. Samples can be safely stored at -20°C and protocol can be continued at a later date.

- Quantify the cDNA using Qubit. Use **1 µl** of clean cDNA for quantification.
- Quality check the cDNA using the Agilent 2100 Bioanalyzer with High Sensitivity DNA Analysis Kits. 或者跑胶看一眼。
- 产物够多次建库的量，稀释至 **4-8 ng/µL** 用于后续 NEB protocol 或者 **0.2 ng/µL** 用于 Nextera protocol

# Library Preparation Using NEB (Decode3/pm)

## NEB Fragmentation

- Prepare Fragmentation Mix

| Reagent | 1x |
|---|---|
| Ultra II FS Reaction Buffer | 1.4 µL |
| Ultra II FS Enzyme Mix | 0.4 µL |
| cDNA (4-8 ng/µL) | 2.5 µL |
| TE | 1.7 µL |

| Reagent | 1x |
|---|---|
| Total | 6 µL |

- 📓 Notes: Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.
- 📓 Notes: Vortex the Ultra II FS Enzyme Mix for 5-8 seconds prior to use for optimal performance.
- Vortex the Fragmentation Mix for 5s and immediately proceed to next step.
- Incubate the Fragmentation reaction as follows:

| Step | Temperature | Time |
|---|---|---|
| Pre-Cool | 4 C | ∞ |
| Fragmentation | 37 C | 5 min |
| A Tailing and Phosphorylation | 65 C | 30 min |
| Storage | 4 C | ∞ |

- 📓 Notes: Set heated lid to 75° C.

## NEB Adapter Ligation

- Prepare Adapter Ligation Mix

| Reagent | 1x |
|---|---|
| NEBNext Ultra II Ligation Master Mix | 6 µL |
| NEBNext Ligation Enhancer | 0.2 µL |
| prime-seq Adapter (1.5 µM) | 0.5 µL |
| Total | 6.7 µL |

- Add **6.7 µl** Adapter Ligation Mix to each replicate
- Incubate for **15min** at 20°C
  - 📓 Notes: Turn off heated lid
- Add **37.3 µl** Buffer EB to Samples

    - Buffer EB - QIAGEN: The composition of Buffer EB is: 10 mM Tris-Cl, pH 8.5。暂用 pH 8.0 Tris-Cl 代替
- Mix Index PCR with **26 µl** SPRI select beads (ratio of **1:0.52**)
    - 📓 Notes: $26/50 = 0.72$
    - 📓 Notes: We use **SPRI Select Beads** here instead of our home made 22% Cleanup beads for their guaranteed QCed size selection properties.
- Incubate for 5min at 20°C (Room Temp)
- Place the plate on the magnet stand until clear and transfer 76 µl supernatant to clean well.
- Mix supernatant with **10 µl** SPRI select beads (ratio of **1:0.72**)
    - 📓 Notes: $(26 + 10/50) = 0.72$
- Incubate for 00:05:00 at 20°C (Room Temp)
- Place the plate on the magnet stand until clear and discard supernatant.
- Wash with 150 µl of 80% EtOH while the plate is on the magnet. Discard the supernatant
- Repeat wash step once more
- Air dry beads for 5min
- Elute samples in **10.5 µl** 0.1X TE (dilute 1X TE Buffer 1:10 in water) for 5min

# NEB Library PCR

- Transfer samples to clean wells
- Add **1µl** of i7 Index Primer (5 uM) to each well
    - 📓 Notes: This is the unique index that will be used for demultiplexing libraries.
- Prepare Library PCR Mix

| Reagent | 1x | 5x |
|---|---|---|
| NEBNext Ultra II Q5 Master Mix | 12.5 µL | 62.5 µL |
| P5R1/pm-3enrich (5uM) ⭐ | 1 µL | 5 µL |
| Total | 13.5 µL | 67.5 µL |

- 📓 Notes: Although scaled down, there will not be sufficient **Q5 Master Mix** (M0544S 1.25ml /M0544L 6.25ml) in the kit. This item will have to be ordered separately.
- 📓 FAQ: What is the difference between NEBNext Ultra II Q5 Master Mix and NEBNext High-Fidelity 2X PCR Master Mix (M0541)? Although both master mixes contain Q5 High-Fidelity DNA Polymerase, the master mix composition is different. NEBNext Ultra II Q5 Master Mix has been specifically optimized for to faithfully amplify NGS libraries with the least amount of amplification bias. Additionally, amplification of NGS libraries in the presence or absence of magnetic beads is more robust, and it is a hot start, making it ideal for workflows involving automation.

- Add **13.5µl** of Library PCR Mix to each well
- Incubate the Library PCR reaction as follows:

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Initial Denaturation | 98 C | 30 sec | 1 cycle |
| Denaturation | 98 C | 10 sec | 10 cycles* |
| Annealing/Elongation | 65 C | 1 min 15 sec | |
| Final Elongation | 65 C | 5 min | 1 cycle |
| Storage | 4 C | hold | |

- Adjust the number of cycles based on cDNA input. As a general guide we recommend.

| cDNA Input | Cycles |
|---|---|
| 20 ng | 10 |
| 10 ng | 11 |
| 5 ng | 12 |

# Library Double Size Selection (Decode3/pm)

- Add **25 µl** Buffer EB to Index PCR (补齐PCR体系体积至50µL)
- Mix Index PCR with **26 µl** SPRI select beads (ratio of **1:0.52**)
- Incubate for 00:05:00 at 20°C (Room Temp)
- Place the plate on the magnet stand until clear and transfer **76 µl** supernatant to clean well.
- Mix supernatant with **10 µl** SPRI select beads (ratio of **1:0.72**)
- Incubate for 5min at 20°C (Room Temp).
- Place the plate on the magnet stand until clear and discard supernatant.
- Wash with 150 µl of 80% EtOH while the plate is on the magnet. Discard the supernatant.
- Repeat wash step once more.
- Air dry beads for 5min.
- Elute in **15 µl** UltraPure Water.
- Incubate for 5min and then place on magnet until clear. Transfer eluted library to new well.