**Protocol Overview**

**mRNA-seq and ribosome profiling**

**Cell harvest and lysis**

-capture cells through vacuum filtration

-lyse cells using sheer force via mixer mill

-collect cell lysate

**mRNA purification**

-extract RNA from lysate using phenol/chloroform

-subtract short RNAs using MEGAClear kit (optional)

-subtract rRNAs using MicrobEXPRESS kit (optional)

-alkaline fragmentation

-gel purify fragmented mRNAs

-merge with profiling samples for library preparation

**Ribosome purification**

-digest lysate to enrich monosomes

-ultracentrifuge digested lysate on sucrose gradient

-fractionate gradients and collect monosome peaks

-extract RNA from monosomes using phenol/chloroform

-gel purify monosome protected fragments

-merge with mRNA samples for library preparation w/ exception:

-subtract rRNA after circles are made

**Library preparation**

-dephosphorylate 20pmol of sample

-ligate on linker-1

-gel extract linked products

-RT linked products to generate ssDNA

-gel extract RT product from excess RT oligo

-circularize clean RT product

-ribo prep ONLY: subtract out rRNA circles

-PCR amplify off circles to create sequencing library

-run Bioanalyzer chip

**Cell Harvest and Lysis**

Cell Harvest

1) Grow culture to desired OD

*example: 200mL WT culture in MOPS-complete+Glu to OD420=0.5*

2) Place harvesting set-up at growth temperature, ie 37C room

-9mm nitrocellulose filters

-tweezers and spatulas

-filter unit and waste collection

-vacuum pump

-sterile bottle of liquid nitrogen(lN2)

-50mL conical for each sample

3) Pour culture on filter, pipette out 1mL for OD reading later

*should take about 60 seconds to flow through*

4) Set double-sided spatula in 50mL conical and fill with liquid nitrogen

5) Once culture is completely filtered, look for excess media on the edges of filter apparatus, break vacuum at filter flask, remove filter clamp, remove top of the filter unit, and scrape off cell paste using a flat-sided spatula

6) Immediately drop cell paste spatula into conical of lN2 and use second pre-cooled spatula to remove all cell paste into the conical tube

7) Place 50mL conical with cell paste and lN2 into -80C freezer with cap ajar allowing lN2 to boil off

8) Once lN2 has boiled off continue to cell lysis. Samples can be left in -80C freezer O/N until mixer milling

Cell lysis and rough purification

Make chloramphenicol at 50mg/mL in etOH for every experiment, store at 4C

1) Make cell re-suspension buffer

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 1M NH4Cl | 60.0uL |  |
| 1M MgCl2 | 6.0uL |  |
| 1M Tris pH 8.0 | 12.0uL |  |
| NP-40 | 0.6uL |  |
| Triton X-100 | 2.4uL |  |
| 50mg/mL chloramp. | 3.9uL |  |
| DNase I | 6.5uL |  |
| DEPC water | 565uL |  |

2) Wash 10mL mixer mill canisters with milli-Q water, etOH, and again in water. Dry thoroughly paying special attention to threading of the canisters

3) Place ball bearing in large side of canister, and submerge both halves into lN2, cool until canisters are no longer boiling

4) Add 600uL cell re-suspension buffer and cell paste to the large side of canister, record which sample is in each canister. Place back into lN2

5) Mixer mill at 15Hz for 3 min, repeat for a total of 5 rounds, re-cooling in lN2 after each round

6) Fill a 50mL conical with lN2, also cool a double sided spatula

7) Scrape cells out of the canister[[1]](#footnote-1) and into the lN2, place in -80C freezer until lN2 has boiled off. Samples can be left at -80C O/N

8) Thaw cell lysate powder in 30C water bath for 2 min, then on ice for 15 min

9) Transfer cell lysate to 1.5mL tube and spin at 14000rpm for 10 min at 4C. Move supernatant to fresh 1.5mL tube, avoiding top white layer and pellet

11) Mix tube by swirling and make a 1/50 dilution in 10mM Tris to measure A260. Vortex well.

*example calculation:*

|  |  |  |
| --- | --- | --- |
|  | 1/50 A260 | A260/mL |
| SampleA | 3.9 | 195 |

12) Aliquot: 200uL for ribosomes, 40uL mRNA. Snap freeze in lN2 and store at -80C.

**mRNA purification**

Phenol chloroform RNA extraction

45 min + precipitation: 1.5 hrs

*Note: This RNA extraction can be completed simultaneously with pooled monosome samples to save time.*

1) Combine 30uL cell lysate and 670uL of 10mM Tris to fresh 1.5mL tube, add 80uL of 10% SDS to each sample

2) Set Thermomixer to 65C and prewarm 750uL phenol:chloroform for each sample.

3) Add 750uL hot phenol:chloroform to each sample, incubate 5 min at 65C with constant shaking. Place on ice for 5 min, spin 5 min at 14000rpm at 4C

5) Transfer supernatant to tube containing 700uL phenol:chloroform , incubate 5 min at RT with occasional vortexing. Spin 5min at 14000rpm at 4C

6) Transfer supernatant to tube containing 600uL chloroform, vortex for 30 sec at RT. Spin 5min at 14000rpm at 4C

7) Transfer supernatant to fresh 1.5mL tube and precipitate:

55uL 3M NaOAc

2uL GlycoBlue

550uL isopropanol

8) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet.

9) Resuspend in 30uL 10mM Tris if proceeding to MEGAclear short RNA subtraction

**mRNA purification**

MEGAclear kit: short RNA subtraction[[2]](#footnote-2)

15 min + precipitation: 1.5 hr

1) Preheat 100uL DEPC water per sample in 95C heat block

2) Add 20uL sample to a 1.5mL tube, add 80uL kit elution solution to bring each sample to 100uL and mix

3) Add 350uL binding solution, mix by pipetting

4) Add 250uL 100% etOH, mix by pipetting

5) Apply full sample to filter cartridge and spin at 14000rpm for 30 sec

*note: this kit was historically problematic and some save ALL flow-through in this protocol to precipitate later in case of catastrophic RNA loss*

6) Wash 2x with 500uL wash solution, spin 10 additional seconds to remove all wash

7) Elute RNA by adding 50uL of 95C water to filter, wait 1 min, spin at 14000rpm for 1 min. Repeat this elution step into same collection tube.

8) Precipitate elution in a fresh 1.5mL tube

11uL 3M NaOAc

2uL GlycoBlue

100uL isopropanol

9) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet.

10) Re-suspend in 15uL 10mM Tris, and nanodrop

**mRNA purification**

MicrobEXPRESS: rRNA subtraction[[3]](#footnote-3)

1.5 hr + precipitation: 3 hr

Typically this protocol is run in pairs of samples offset by at least 10min to sure properly timed captures

1) Add 9ug of sample to a 1.5mL tube containing 200uL binding buffer, vortex

2) Add 4uL capture oligo to each tube, vortex briefly then microfuge

3) Heat at 70C for 10 min, then add 2uL SuperaseIN

4) Anneal at 37C for 45 min

Prepare magnetic beads for capture during anneal incubation

5) Add 50uL of each oligo MagBeads per sample to a 1.5mL tube

6) Place tube on magnetic stand, incubate at RT for 3 min. Remove supernatant

7) Add equivalent volume (50uL/rxn) of water, remove from stand and briefly vortex

8) Recapture beads on magnetic stand as described above, then remove sup.

9) Add equivalent volume (50uL/rxn) of binding buffer, remove from stand and re-suspend by gentle vortex.

10) Recapture beads on magnetic stand as described above, then remove sup.

11) Add equivalent volume (50uL/rxn) of binding buffer again, re-suspend, then equilibrate beads at 37C

Capture rRNA and recover mRNA

12) Heat wash solution to 37C

13) Add 50uL magnetic beads to sample, incubate at 37C for 15min

14) Capture beads on stand for 3 min, remove sup to collection tube. Add 100uL wash solution to captured beads, re-suspend and recapture. Pool sup with prior collection. Repeat wash solution capture

15) Precipitate: 44uL 3M NaOAc, 2uL GlycoBlue, 440uL isopropanol

**mRNA purification**

Alkaline fragmentation (using Ambion RNA fragmentation kit)

10 min + precipitation: 1.5 hr

1) Re-suspend sample in 40uL 10mM Tris pH 7.0

2) Incubate 2 min at 95C in PCR block, place on ice

3) Add 4.4uL 10X fragmentation buffer

4) Fragment 1 min 45 sec in 95C PCR block

5) Add 5uL stop buffer

6) Add 100uL 10mM Tris pH 7.0 prior to precipitation

15uL 3M NaOAc

2uL GlycoBlue

200uL isopropanol

7) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet.

8) Re-suspend in 8uL 10mM Tris pH 7.0

**mRNA purification**

Gel purify and size selection of fragmented RNA

1 hr + gel running + precipitation: 3 hr

1) Set up 15% TBE-Urea gel in 1X TBE, pre-run 1 hr at 200V, rinse wells

2) Add 8uL 2x denaturing loading dye to each sample

3) Dilute 1uL of 10bp ladder in 15uL of 10mM Tris, add 15uL 2x loading dye

4) Denature all samples and ladder at 80C for 1 min, return to ice

5) Load samples, skipping lanes between each.

6) Run for 65min at 200V, stain gel with 1uL Sybr-Gold in 100mL 1X TBE for 3minutes, photograph gel, excise 25-45bp or desired size

RNA gel recovery solution

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 20U/uL SuperaseIN | 2uL |  |
| 3M NaOAc | 40uL |  |
| 0.5M EDTA | 0.8uL |  |
| DEPC water | 360uL |  |

*note: alternatively use water, but remember to add salts before precipitation*

7) Place excised gel slice in a pierced bottom 0.5mL tube, nested in a 1.5mL tube

*note: pierce bottom of 0.5mL tube using a 20-gauge needle*

8) Spin nested tubes at max speed for 3 min at RT

9) Collect any remaining gel in 0.5mL tube, combine with crushed gel

10) Add 200uL of gel recovery solution to crushed gel, incubate at 70C for 10 min at 1400rpm on Thermomixer

11) Cut the tip off a p1000 tip and transfer gel slurry to spin-X cellulose acetate column. Spin column at max speed for 2 min at RT

12) Transfer flow through to a fresh 1.5mL tube. Precipitate with 2uL GlycoBlue

and 300uL isopropanol

7) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet. Resuspend in 15uL 10mM Tris pH 7.0

**Ribosome purification**

Digest cell lysate to enrich monosomes

1.5 hr

Make fresh 1M DTT for every experiment, store at 4C

1) Make 100mL sucrose buffer for digestions and gradients, combine following reagents. Split into two 50mL conicals, fill to 50mL with DEPC water

|  |  |  |
| --- | --- | --- |
|  | Volume (100mL) | Final [ ] |
| 1M NH4Cl | 15mL | 100mM |
| 1M MgCl2 | 1.5mL | 10mM |
| 1M CaCl2 | 750uL | 5mM |
| 1M Tris pH 8.0 | 3mL | 20mM |
| 50mg/mL chloramp | 150uL | 100ug/mL |
| 1M DTT | 300uL | 3mM |

2) Make a digestion master mix (MM)

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 100mM CaCl2 | 6.7uL |  |
| SuperaseIN | 6.7uL |  |
| 50mg/mL chloramp | 0.4uL |  |

3) Set up digestion reactions with 14 A260 units RNA for digested samples, and 4 A260 units for controls

*example set-up:*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Reaction name | MM(uL) | Lysate(uL) | MNase(uL) | Sucrose buffer (uL) |
| WT | 13.8 | 63 | 2.3 | 120.9 |
| dRF3 | 13.8 | 141 | 2.3 | 42.9 |
| RF2-Bstrain | 13.8 | 74.8 | 2.3 | 109.1 |
| dRF3- RF2-B | 13.8 | 69.2 | 2.3 | 114.7 |
| WT control | 13.8 | 16.7 | - | 167.2 |
| dRF3 control | 13.8 | 37.7 | - | 146.2 |

4) Digest samples at 25C for 1 hour

5) Quench reactions with 2.4uL EGTA, place on wet ice until loaded on sucrose gradients or snap freeze in lN2 and store at -80C

**Ribosome purification**

Ultracentrifugation of digested products

1hr + spin time: 3.5 hr

1) Create sucrose solutions for gradient making, then briefly centrifuge to remove bubbles, 2 min at 2000rpm

55% sucrose:

22g sucrose, fill to 40mL with sucrose buffer

10% sucrose:

4g sucrose, fill to 40mL with sucrose buffer

2) Pipette 6mL of 55% sucrose solution to the bottom of an ultracentrifuge tube. Then gently pipette 6mL of 10% solution on top of the heavier solution, avoiding bubbles or disruption of meniscus

3) Place rubber tube caps on each tube, rolling them on towards the air-release hole in the rubber cap. The release hole is marked with a vertical line on the side of the cap, this will ensure no bubbles are trapped in the gradient

3) Create gradients by placing tubes onto tube holder, select “GMST” on machine, then “last” for a 7%-47% gradient

*note: these gradients can store O/N at 4C*

4) Place gradients inside SW41 tube holders and balance tube pairs to within 0.1g using 10% sucrose.

5) Add digested samples to the top of the gradients. Pipette on the side of the tube, allowing the sample to spread across the top without disrupting the gradient. Check balance again before running ultracentrifuge

5) Run ultracentrifuge, SW41 rotor, at 35000rpm for 2.5 hr at 4C

**Ribosome purification**

Fractionation of gradients

1 hr

1) While ultracentrifuge is running prepare for fractionation. Tools include; 1.5mL tube for each sample on ice, tweezers, wrench for opening rotor tubes, DEPC water, and a blank tube.

2) To begin collecting fractions, turn on gradient machine and UV detector box

3) On gradient machine select “Frac”, “Frac”, “Frac”. This will allow you to move the collector. Wash 2x with DEPC water.

4) Load sample tube, move collector to top of visual field, select “Frac” then reset the position of the collector.

5) Again select “Frac” then “Sing”. Adjust “SPED” to 0.2, “DIST” to >90, “NUM” to 1. This sets the speed and depth the collector will go when the start button is pressed

6) On the computer open DATAQ, start Windaq, select “file” then “record” to save a new file. Once file name is entered, a new screen with “OK” will appear. Select OK on computer and Start on the gradient machine simultaneously

7) 9 seconds (this may change if tubing is altered, check machine for the most recent number) after monosomes appear on screen, collect the sample ~1.2mL.

8) Once the collector has run through the sample tube, select “file” then “stop” and then “file” then “close”. If run isn’t stopped then closed data cannot be accessed.

9) Data files can be exported by opening file after run has been closed out, then selected “Save As” and changing .txt to .xls

*note: pooled monosomes can be stored at -80C until use*

**Ribosome purification**

Phenol chloroform extraction of RNA from pooled monosomes

45 min + precipitation: 1.5 hr

1) Combine 700uL of pooled monosomes with 80uL of 10% SDS to each sample

2) Set Thermomixer to 65C and prewarm 750uL phenol:chloroform for each sample.

3) Add 750uL hot phenol:chloroform to each sample, incubate 5 min at 65C with constant shaking. Place on ice for 5 min, spin 5 min at 14000rpm at 4C

5)Transfer supernatant to tube containing 700uL phenol:chloroform , incubate 5 min at RT with occasional vortexing. Spin 5min at 14000rpm at 4C

6) Transfer supernatant to tube containing 600uL chloroform, vortex for 30 sec at RT. Spin 5min at 14000rpm at 4C

7) Transfer supernatant to fresh 1.5mL tube and precipitate:

55uL 3M NaOAc

2uL GlycoBlue

550uL isopropanol

8) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet.

9) Resuspend in 8uL 10mM Tris pH 7.0

**Ribosome purification**

Gel purify monosome protected fragments

1 hr + gel running + precipitation: 3 hr

1) Set up 15% TBE-Urea gel in 1X TBE, pre-run 1 hr at 200V, rinse wells

2) Add 8uL 2x denaturing loading dye to each sample

3) Dilute 1uL of 10bp ladder in 15uL of 10mM Tris, add 15uL 2x loading dye, also dilute sizing oligos to same concentration if desired

4) Denature all samples and ladder at 80C for 1 min, return to ice

5) Load samples, skipping lanes between each.

6) Run for 65min at 200V, stain gel with 1uL Sybr-Gold in 100mL 1X TBE for 3minutes, photograph gel, excise 28mers or desired size (sizing oligos 26bp-30bp)

RNA gel recovery solution

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 20U/uL SuperaseIN | 2uL |  |
| 3M NaOAc | 40uL |  |
| 0.5M EDTA | 0.8uL |  |
| DEPC water | 360uL |  |

*note: alternatively use water, but remember to add salts before precipitation*

7) Place excised gel slice in a pierced bottom 0.5mL tube, nested in a 1.5mL tube

*note: pierce bottom of 0.5mL tube using a 20-gauge needle*

8) Spin nested tubes at max speed for 3 min at RT

9) Collect any remaining gel in 0.5mL tube, combine with crushed gel

10) Add 200uL of gel recovery solution to crushed gel, incubate at 70C for 10 min at 1400rpm on Thermomixer

11) Cut the tip off a p1000 tip and transfer gel slurry to spin-X cellulose acetate column. Spin column at max speed for 2 min at RT

12) Transfer flow through to a fresh 1.5mL tube. Precipitate with 2uL GlycoBlue

and 300uL isopropanol

7) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet. Resuspend in 15uL 10mM Tris pH 7.0

**Library preparation**

Dephosphorylation[[4]](#footnote-4)

1 hr + precipitation: 2 hr

1) Dilute 20pmol (~200ng) of each sample in 7uL of 10mM Tris pH 7.0

*note: less starting product can be used and subsequent reactions scaled*

2) Set heat blocks for 70C and 37C

3) Add 1uL PNK buffer to each sample and denature at 70C for 1 min, return to ice

4) Add 1uL SuperaseIN and 1uL t4 PNK to each sample, mix well by pipetting

5) Incubate at 37C for 1 hr, then heat inactivate at 65C for 3 min

*note: heat inactivation is optional, but generally performed.*

See appendix for 2-step de-phosphorylation and ligation protocol if desired

Ligation of linker-1 to sample

3.5 hr + precipitation: 4.5 hr

1) To each sample add:

7uL 50% PEG8000

1uL 10X T4 ligase buffer

1uL 10mM DTT (fresh)

1uL 100uM linker-1

4uL 1:20 purified T4 ligase

2) Mix thoroughly with pipette, really well mixed

3) Incubate at 25C for 2.5-3.5 hr

4) Add 100uL 10mM Tris pH 7.0 to bring up volume then precipitate

12uL 3M NaOAc

2uL GlycoBlue

200 isopropanol

5) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet.

6) Resuspend in 8uL 10mM Tris pH 7.0

**Library preparation**

Gel purify 3’ linked fragments

1 hr + gel running + precipitation: 3 hr

1) Set up 10% TBE-Urea gel in 1X TBE, pre-run 1 hr at 200V, rinse wells

2) Add 8uL 2x denaturing loading dye to each sample

3) Dilute 1uL of 10bp ladder in 15uL of 10mM Tris, add 15uL 2x loading dye, also dilute sizing oligos to same concentration if desired

4) Denature all samples and ladder at 70C for 1 min, return to ice

5) Load samples, skipping lanes between each

6) Run for 55min at 200V, stain gel with 1uL Sybr-Gold in 100mL 1X TBE for 3minutes, photograph gel, excise linked product from unlinked product and excess linker. Linker-1 is 17bp long so expected profiling product is ~40-50bp.

RNA gel recovery solution

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 20U/uL SuperaseIN | 2uL |  |
| 3M NaOAc | 40uL |  |
| 0.5M EDTA | 0.8uL |  |
| DEPC water | 360uL |  |

*note: alternatively use water, but remember to add salts before precipitation*

7) Place excised gel slice in a pierced bottom 0.5mL tube, nested in a 1.5mL tube

8) Spin nested tubes at max speed for 3 min at RT

9) Collect any remaining gel in 0.5mL tube, combine with crushed gel

10) Add 200uL of gel recovery solution to crushed gel, incubate at 70C for 10 min at 1400rpm on Thermomixer

11) Cut the tip off a p1000 tip and transfer gel slurry to spin-X cellulose acetate column. Spin column at max speed for 2 min at RT

12) Transfer flow through to a fresh 1.5mL tube. Precipitate with 2uL GlycoBlue

and 300uL isopropanol

7) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet. Resuspend in 10uL 10mM Tris pH 7.0

**Library preparation**

RT linked fragments to make ssDNA

1 hr

1) Add 1uL 10mM dNTPs and 1.5uL 5uM RT primer to each sample

2) Denature at 65C for 5 min then 45C for 5 min in PCR block, then ice

*note: set up a PCR block with all conditions for RT, pausing between steps*

3) Make RT master mix while incubating

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| First Strand Buffer | 4uL |  |
| SuperaseIN | 1uL |  |
| 0.1M DTT | 1uL |  |

4) Add 6uL of master mix to each sample on ice, then add 1uL Superscript III and mix throughly

5) Incubate 50C for 30min

6) Add 2.3uL 1N NaOH to hydrolyze remaining RNA, incubate 95C for 15 min

7) Store at -80C or continue to gel purification

**Library preparation**

Gel purification of RT linked fragments

1 hr + gel running + precipitation: 3 hr

1) Set up 10% TBE-Urea gel in 1X TBE, pre-run 30 min at 200V, rinse wells

2) Add 23uL 2x denaturing loading dye to each sample

3) Dilute 1uL of 10bp ladder in 15uL of 10mM Tris, add 15uL 2x loading dye

4) Denature all samples and ladder at 80C for 1 min, return to ice

5) Load samples, splitting sample in 2 lanes and skipping a lane between samples

6) Run for 80min at 200V, stain gel with 1uL Sybr-Gold in 100mL 1X TBE for 3minutes, photograph gel, excise 28mers or desired size

DNA gel recovery solution

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 4M NaCl | 30uL |  |
| 0.5M EDTA | 0.8uL |  |
| 10mM Tris pH 8.0 | 400uL |  |

*note: alternatively use water, but remember to add salts before precipitation*

7) Place excised gel slice in a pierced bottom 0.5mL tube, nested in a 1.5mL tube

8) Spin nested tubes at max speed for 3 min at RT

9) Collect any remaining gel in 0.5mL tube, combine with crushed gel

10) Add 200uL of gel recovery solution to crushed gel, incubate at 70C for 10 min at 1400rpm on Thermomixer

11) Cut the tip off a p1000 tip and transfer gel slurry to spin-X cellulose acetate column. Spin column at max speed for 2 min at RT

12) Transfer flow through to a fresh 1.5mL tube. Precipitate with 2uL GlycoBlue

and 300uL isopropanol

7) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet. Resuspend in 15uL 10mM Tris pH 8.0

**Library preparation**

Circularize ssDNA

2.5 hr

1) Make the circularization master mix:

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| CircLigase Buffer | 2uL |  |
| 1mM ATP | 1uL |  |
| 50mM MnCl2 | 1uL |  |

2) Add 4uL of master mix to each 15uL sample, mix

3) Add 2uL CircLigase to each sample and incubate at 60C for 2 hrs

4) Heat inactivate at 80C for 10 min, freeze at -20C

**Library preparation**

For ribosome samples only:

Subtraction of rRNA circles

1.5 hr + precipitation: 2.5 hr

1) Make 5mL of each 1X and 2X bind and wash buffer (B&W)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | 1X | 2X | Final [1x] | Final [2x] |
| 2M Tris pH 7.5 | 12.5uL | 25uL | 5mM | 10mM |
| 500mM EDTA | 5uL | 10uL | 0.5mM | 1mM |
| 4M NaCl | 1.3mL | 2.5mL | 1M | 2M |
| Tween | 0.5uL | 1.0uL | 0.01% | 0.02% |

2) Aliquot 5uL of circularized DNA for each sample in a PCR tube

3) Make oligo subtraction master mix

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 1055-58 oligo mix | 1uL |  |
| 20X SSC | 1uL |  |
| DEPC | 3uL |  |

4) Add 5uL of oligo master mix to each sample and incubate at 98C for 75 sec in PCR. Ramp down to 37C over 1hr (1C/min from 97C). Then 37C for 20 min.

5) Prepare magnetic beads during incubation, 25uL of MyOne Streptavidin C1 Dynabeads per sample

6) Wash beads 3x with equivalent volume of 1X B&W buffer. Place beads on magnetic stand, wait 3 min, remove sup and discard. Repeat.

7) Resuspend in 10uL of 2X B&W buffer per sample, place beads at 37C

8) After oligo incubation, add 10uL washed beads. Incubate at 37C for 15min.

9) Capture beads on magnetic stand, remove eluate and precipitate out DNA

10) Add 100uL 10mM Tris pH 8.0 to bring up volume then precipitate

12uL 4M NaCl

2uL GlycoBlue

1.5uL 0.5M EDTA

200uL isopropanol (etOH leaves too much salt for subsequent PCR)

5) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet. Resuspend in 10uL 10mM Tris pH 8.0

**Library preparation**

PCR amplify off circles

1 hr

1) Aliquot 5uL of DNA into fresh tube

2) Make master mix, add 75uL of mix to each sample

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 5X HF buffer | 16.7uL |  |
| 10mM dNTPs | 1.7uL |  |
| 10uM ADP-1 primer | 4uL |  |
| DEPC water | 51.5uL |  |
| HF Phusion | 1uL |  |

3) Add 4uL 10uM of multiplexing oligo to each sample and mix thoroughly. Use a different oligo for each sample

4) Aliquot 17uL of sample mix into 4 separate PCR strips. Each strip will be taken out at a different number of cycles

5) Run PCR program:

98C :30

98C :10 |

60C :10 | 14 cycles

72C :05 |

72C :30

4C hold

6) Pull out a strip of samples at 4 cycle conditions. Either at 6, 8, 10, 12 or 8, 10, 12, 14

7) Add 3.5uL of 6X DNA loading dye

**Library preparations**

Gel purify PCR products

1 hr + gel running + precipitation: 3 hr

1) Set up 8% TBE gel in 1X TBE, rinse wells and load samples

6) Run for 50 min at 180V, stain gel with 1uL Sybr-Gold in 100mL 1X TBE for 3minutes, photograph gel, excise product

DNA gel recovery solution

|  |  |  |
| --- | --- | --- |
|  | 1X |  |
| 4M NaCl | 30uL |  |
| 0.5M EDTA | 0.8uL |  |
| 10mM Tris pH 8.0 | 400uL |  |

*note: alternatively use water, but remember to add salts before precipitation*

7) Place excised gel slice in a pierced bottom 0.5mL tube, nested in a 1.5mL tube

8) Spin nested tubes at max speed for 3 min at RT

9) Collect any remaining gel in 0.5mL tube, combine with crushed gel

10) Add 200uL of gel recovery solution to crushed gel, incubate at 70C for 10 min at 1400rpm on Thermomixer

11) Cut the tip off a p1000 tip and transfer gel slurry to spin-X cellulose acetate column. Spin column at max speed for 2 min at RT

12) Transfer flow through to a fresh 1.5mL tube. Precipitate with 2uL GlycoBlue

and 300uL isopropanol

7) Incubate in -80C for 30 min or dry ice for 15 min. Spin max speed for 30 min at 4C. Wash pellet with 750uL 80% etOH, pulse spin, remove etOH and dry pellet. Resuspend in 15uL 10mM Tris pH 8.0

**Bioanalyzer**

Set-up and running of BioAnalyzer chip

1.5 hr

1) Back dilute samples 1uL in 5uL of 10mM Tris pH 8.0

2) Allow gel-matrix in kit to equilibrate to RT for 30 min

3) Follow kit instructions on loading and running BA chip

Pooling samples using BioAnalyzer data

Usually we submit 15uL of 2nM pooled sample, this is equal to 30fmol of total product

1) Calculate the number of fmol per sample needed by dividing the total number of fmol by the number of samples you are combining in a lane

2) Using the concentration (pmol/L) given by the BioAnalyzer peaks we can calculate our concentration in fmol/uL for each sample. Remember to account for your dilution factor, generally 4 or 5

3) Use this samples concentration in fmol/uL to determine how many microliters of each sample you need to reach the appropriate number of fmol calculated in step 1

4) After combining the volume needed from each sample in step 3, bring to final volume with the addition of DEPC water.

**Appendix**

Two-step dephosphorylation and ligation

1) After dephosphorylation, precipitate reaction and resuspend in 5uL 10mM Tris pH 7.0

2) For each sample add:

8uL 50% PEG-8000

2uL DMSO

2uL 10X T4 ligase buffer

1uL SuperaseIN

1uL 100uM linker-1

2uL 1:20 purified T4 ligase

3) Proceed with incubation at 25C for 2.5-3.5 hr, then precipitate and proceed to gel purification

Excess linker-1 digestion protocol

Making rRNA circle subtraction oligo mix:

|  |  |  |
| --- | --- | --- |
| **100uM stock** | **100uL 55/58 mix** | **Sequence** |
| o1055 | 77uL | 5'\_5Biosg/TCATCTCCGGGGGTAGAGCACTGTTTCG\_3 |
| o1056 | 4uL | 5'\_5Biosg/GGCTAAACCATGCACCGAAGCTGCGGCAG\_3' |
| o1057 | 17uL | 5'\_5Biosg/AAGGCTGAGGCGTGATGACGAGGCACT\_3' |
| o1058 | 2uL | 5'\_5Biosg/CGGTGCTGAAGCAACAAATGCCCTGCTT\_3' |

1. Use the spoon end of the spatula to scrape up cell powder, then quickly slide down the side of the conical tube to plunge powder into lN2 [↑](#footnote-ref-1)
2. The manual for the MEGAclear Ambion kit can be found online [↑](#footnote-ref-2)
3. The manual for the MicrobEXPRESS kit can be found online [↑](#footnote-ref-3)
4. Less than 200ng of sample can be used, closer to 50ng. If using less than 200ng reduce amount of linker-1 and RT oligo used in subsequent steps. [↑](#footnote-ref-4)