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Whole-genome STARR-seq Protocol – Reddy Lab 2023

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We use this protocol and it's working

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Abstract

Reddy lab whole genome STARR-seq protocol. The document includes the input, transfection and output library generation, which should be the expected protocol products. Adapted from the document found in STARR-seq experiment from the Reddy lab in the ENCODE project (document attached with additional details).

Attachments

PDF

wgSTARRseq_ENCODE_KP...

230KB

Materials

- NEBNext DNA Library Prep Master Mix Set for Illumina - 60 rxns **New England Biolabs Catalog #**E6040L
- KAPA HiFi HotStart PCR Kit (Cat. # KK2502)
- SYBR™ Safe DNA Gel Stain **Thermo Scientific Catalog #**S33102
- GeneJET Gel Extraction Kit **Thermo Fisher Catalog #**K0692
- NEBuilder HiFi DNA Assembly Master Mix **New England Biolabs Catalog #**E2621
- MN Nucleobond PC 10000 EF (Cat. #740548)
- RNase Block **Agilent Technologies Catalog #**300151
- Dynabeads mRNA Purification Kit (Cat.# 61005)
- TURBO DNA-free™ Kit **Thermo Scientific Catalog #**AM1907
- SuperScript III system (Invitrogen Cat.#18080)
- Ambion™ Recombinant RNase A **Thermo Fisher Scientific Catalog #**AM2269

Part 1. INPUT LIBRARY GENERATION

18h 25m

1 Day 1: Prepare STARR-seq inserts

1.1 Shear DNA to ~500 bp.

1.2 Using 8 rxns with 5 µg of DNA per rxn (total 40 µg DNA per genome), follow the NEBNext DNA Library Prep Master Mix Set for Illumina (Cat. #E6040L) through the End - Repair and A-tailing steps.

1.3 Perform the ligation step with [M] 15 micromolar (µM) STARR-seq adaptors instead of the supplied adaptor (final concentration [M] 3 micromolar (µM)).

Note

STARR-seq adaptors:
Adaptor1: ACACTCTTTCCCTACACGACGCTCTTCCGATC*T
Adaptor2: [Phos]GATCGGAAGAGCACACGTCTGAACTCCGATC*T

1.4 Following the first SPRI cleanup after the ligation step, perform the 500 bp insert SPRI size selection. Elute with 30 µL of EB and collect 27 µL of sample.

1.5 Using the KAPA HiFi HotStart PCR Kit (Cat. # KK2502) amplify the STARR-seq library:

- Primers: TS-2-SS-F + TS-2-SS-R
- Reaction conditions per sample:

	A	B
		ul per sample
	HiFi polymerase	1
	GC 5x buffer	10
	10 uM primer mix	5 + 5

	A	B
	10 mM dNTPS	1.5
	DNA library	25
	H2O	2.5
	Sum	50

- Cycling conditions:

	A	B	C
	95°C	3 min	
	98°C	20 s	10 cycles
	63°C	15 s	
	72°C	1 min	
	72°C	5 min	
	4°C	Hold	

1.6 Pool enriched libraries and perform a 0.9x SPRI cleanup. Elute in 100 µL.

1.7 Assess size distribution on the Tape Station and concentration with Qubit Assay.

1.8 Store at -20 °C.

2 Day 2: Linearize STARR-seq Vector

> **Note**
>
> If using previously linearized vector, run on a gel to check for degradation and double check concentration on Qubit.

2.1 Digest suitable amount of supercoiled STARR-seq plasmid with AgeI and SalI.

2.2 Run restriction products on a large 1% agarose gel and 1kb ladder.

2.3 Stain gel with SybrSafe (Thermo #S33102) on an orbital shaker, covered with foil.

2.4 Excise large band (~2500 bp) with clean razor blade and sterile tweezers.

2.5 Recover fragment with GeneJet Gel Extraction Kit (Thermo #K0692).

2.6 Assess size distribution on the Tape Station and concentration with Qubit Assay.

3 Day 3: Gibson Assembly

3.1 Pre-warm thermocycler to 50 °C.

3.2 Mix the following On ice for a [M] 10 nanomolar (nM) Gibson reaction:

- NEBuilder HiFi DNA Assembly Master Mix (Cat. #E2621)
- For whole-genome STARR-seq, use 20 reactions per genome

	A	B
		per reaction
	Total DNA fragments	0.2 pmols
	Insert:Vector molar ratio	3:1
	NEBuilder HiFi DNA Assembly MM	10uL
	H2O	up to 20uL total

3.3 Distribute up to 100 µL of the MM into each well.

3.4 Incubate for 00:15:00 - 00:30:00 at 50 °C . 30m

3.5 Immediately place the reactions On ice and add EDTA to [M] 10 millimolar (mM) .

- Ex. 2 µL [M] 0.5 Mass Percent EDTA to 100 µL volume

3.6 Pool sample reactions and begin an EtOH precipitation by adding 0.1X volume [M] 3 Mass Percent NaOAc and 2.5X volume very cold 100% EtOH.

3.7 Incubate at -20 °C Overnight . 8h

4 Day 4: Transformation Gibson product

4.1 Centrifuge samples at 16000 x g, 4°C, 00:30:00 . 30m

4.2 Wash pellet with 500 µL cold 70% EtOH, centrifuge at 16000 x g, 4°C, 00:10:00 . Repeat once. 10m

4.3 Let DNA pellet air dry for a few minutes, then resuspend in 40 µL H2O. Continue with transformation or store at -20 °C .

4.4 Split gibson product across 8 cuvettes per genome, each with 300 µL of in-house grown and frozen Endura electrocompetent cells.

4.5 Transform on the BioRad electroporator with the preset protocol: *E. coli* 2mm 3kV.

4.6 Immediately add 1 mL Lucigen Recovery Media warmed to 37 °C . Wash cuvettes 4X and collect in a tube for a total of 5 mL . Recover by shaking for 1h

01:00:00 at 37 °C .

4.7 Plate diluted culture on carbenicillin plates to determine transformation efficiency (puc19) and estimate library diversity the following day.

4.8 Pool transformations into 1 L LB with carbenicillin (4 cuvettes per flask) and shake for 08:00:00 at 37 °C . 8h

4.9 Pellet transformed cells 5000 x g, 4°C, 00:15:00 and store at -20 °C . 15m

5 Day 5: Harvest plasmid DNAs and prepare QC sequencing libraries

5.1 Harvest the STARR-seq plasmid library using 1 Giga-prep column per 2 flasks.

- MN Nucleobond PC 10000 EF (Cat. #740548)

5.2 Determine product concentration with Qubit assay.

5.3 Amplify STARR-seq input sequencing libraries off of plasmid DNAs using the Kapa HiFi HotStart PCR Kit (Cat.# KK2502).

- Primers: 208 TruSeq primer + i7 Index primer or i5 Index primer + i7 PCR primer
- Reaction conditions per sample:

	A	B	C
		ul per sample	**x5.5**
	20ng plasmid DNA	-	-
	GC buffer	5	27.5
	10 mM dNTPS	0.75	4.13
	10 uM primers	0.75 + 0.75	4.13 + 4.13
	Polymerase	0.5	2.75

	A	B	C
	H2O	to 25uL	to 137.5uL

- Cycling conditions:

	A	B	C
	98°C	45 s	
	98°C	15 s	9 cycles
	64.5°C	30 s	
	72°C	1 m. 10 s	
	72°C	1 m	
	4°C	Hold	

5.4 Perform a double-sided bead cleanup (0.5X, 0.9X). Elute with 30 µL of water.

5.5 Assess size distribution on the Tape Station and concentration with Qubit Assay.

5.6 If running multiple samples, pool and dilute to make 20 µL of a 4nM pool.

5.7 Run a MiSeq sequencing run to estimate library quality and diversity before sequencing deeper.

Part 2. TRANSFECTION & OUTPUT LIBRARY GENERATION 4h 16m

6 **Day 6: Transfection**

6.1 Transfect an appropriate amount of the STARR-seq library into the desired cell type using the Lonza 4D Nucleofector and a recommended kit for that cell line.

6.2 Harvest cells 6 hours post-transfection, add RLT buffer (from Qiagen RNeasy kit) with 1% beta mercaptoethanol, and vortex.

6.3 Flash freeze in 50mL conical tubes and store at -80 °C.

7 Day 7: RNA Isolation

7.1 Pass thawed sample through a 18- to 20-guage needle 10 times.

7.2 Isolate RNAs through Qiagen RNeasy midi-columns, performing the on-column DNase step.

> Note
>
> can increase speed and decrease time - 1min instead of 5min

7.3 Elute twice using 125 µL and 75 µL RNase-Free H2O.

7.4 Quantify RNA concentration with Qubit BR and assess R.I.N. values with RNA Tape.

7.5 Add 1 µL RNase Block (Agilent Cat.# 300151).

7.6 Store at -80 °C.

8 Day 8: RNA-seq Library Construction Pt.1

8.1 Prepare 50 mLs of each capture buffer fresh using nuclease-free stocks

- 2x Binding buffer:

	A	B
	Tris-HCl pH 7.5	20 mM
	LiCl	1.0 M
	EDTA	2 mM

- Wash Buffer B:

	A	B
	Tris-HCl pH 7.5	10 mM
	LiCl	0.15 M
	EDTA	1 mM

- 10 mM Tris-HCl pH 7.5

8.2 Prepare RNA

2m

- Use the Dynabeads mRNA Purification Kit (Cat.# 61005).
- Adjust the volume of 75 µg total RNA to 100 µL with [M] 10 millimolar (mM) Tris-HCl, pH 7.5

> Note
>
> If using less than 75 µg RNA scale bead volume (below; 1.0 mg), but keep other volumes constant.

- Heat RNA to 65 °C for 00:02:00 to disrupt secondary structures. Place On ice .

8.3 Prepare Dynabeads

- Transfer 200 µL (1.0 mg) of resuspended Dynabeads to a micro centrifuge tube.
- Place the tube on the magnet and discard the supernatant.

- Remove tube from the magnet and add 100 µL 2x Binding Buffer.
- Place the tube on the magnet and discard the supernatant.
- Remove tube from the magnet and add 100 µL 2x Binding Buffer to the Dynabeads.

8.4 Isolate mRNA

7m

- Add the total RNA to the Dynabeads/2x Binding Buffer suspension.

> Note
>
> Optimal hybridization conditions are obtained in 2x Binding Buffer added in a 1:1 ratio relative to sample volume.

- Rotate for 00:05:00 at Room temperature or mix occasionally by hand.
- Place the tube on the magnet and discard the supernatant.
- Remove the tube from magnet and wash the mRNA-bead complex twice with 200 µL Washing Buffer B.
- Elute with 100 µL of [M] 10 millimolar (mM) Tris-HCl, pH 7.5. Heat to 75 °C for 00:02:00 and place the tube immediately on the magnet.
- Transfer the eluted mRNA to a new RNase-free tube On ice. Save beads for the next step.

8.5 Wash Dynabeads for second capture

- Add 50 µL 2x binding buffer and 50 µL RNase-Free H2O. Remove supernatant and repeat once.
- Remove supernatant and tube from the magnet.
- Add 100 µL 2x Binding Buffer to beads.

8.6 Perform a second mRNA capture

9m

- Heat the eluted RNA to 65 °C for 00:02:00 to disrupt secondary structures. Place On ice.
- Add the 100 µL of RNA to the Dynabeads suspension.

- Rotate for 00:05:00 at Room temperature or mix occasionally by hand.
- Place the tube on the magnet and discard the supernatant.
- Remove the tube from magnet and wash the mRNA-bead complex twice with 200 µL Washing Buffer B.
- Elute with 40 µL of [M] 10 millimolar (mM) Tris-HCl, pH 7.5. Heat to 75 °C for 00:02:00 and place the tube immediately on the magnet.
- Transfer 40 µL of mRNA to a new RNase-free tube On ice .

8.7 Remove contaminating plasmid and genomic DNA

38m

- Prepare DNase digestion master mix using the Turbo DNase (Ambion # AM1907).

	A	B
		ul per sample
	10× TURBO DNase Buffer	5.0
	TURBO DNase	1.0
	RNase Block	1.0
	H2O	3.0
	Sum:	10 µl

- Add 10.0 µL master mix to each well (40 µL mRNA) for a final volume of 50 µL .
- Incubate at 37 °C for 00:30:00 .
- Add 5 µL resuspended DNase Inactivation Reagent and mix well.
- Incubate 00:05:00 at Room temperature , mixing occasionally.
- Centrifuge at 10000 x g, 00:01:30 .
- Transfer 40 µL RNA to a fresh tube, make sure bead slurry does not carry over.
- Add 35 µL of H2O to each tube/well with beads to perform a back extraction with a final volume of ~50 µl.
- Centrifuge at 10000 x g, 00:01:30 .
- Transfer 27 µL of supernatant to matched supernatant from first spin, make sure bead slurry does not carry over.

- Add 1 µL of Agilent RNase Block (40 units/µl), vortex or pipette to mix for a final volume of 68 µL .
- **Optional:** Quantify RNA and DNA concentration (Qubit). Replace volume with H2O for a final volume of 68 µL .

8.8 Reverse Transcription

2h 20m

- Use the SuperScript III system (Invitrogen Cat.#18080) and a reporter-specific primer (RT_UMI) to perform first-strand cDNA synthesis.

1. Each sample will be primed in a single reaction using the first master mix.
2. In the second reaction, the sample will be split into two unequal amounts; 5/6 (65 µL) will be reverse-transcribed and 1/6 (13 µL) will serve as a no-RT control.

- Prepare first master mix.

	A	B
		µl per sample
	4 uM RT_UMI Primer	3.0
	10 mM dNTPs	6.0
	RNase Block	1.0
	Sum:	10 µl

- Add 10 µL master mix to each RNA sample (~ 68 µL) for a final volume of 78 µL .
- Heat samples to 65 °C for 00:05:00 and incubate On ice for at least 2 minutes.
- From each sample, remove 13 µL to a new well. This is your RT- control.
- Prepare the RT+ master mix.

	A	B
		µl per sample
	5X 1st Strand Buffer	20.0
	0.1 M DTT	5.0

	A	B
	RNase Block	5.0
	SuperScript III (200U/ul)	5.0
	Sum:	35 µl

- Add 35 µL master mix to each primed RT+ sample (65 µL) for a final volume of 100 µL .
- Prepare the RT- master mix.

	A	B
		µl per sample
	5X 1st Strand Buffer	4.0
	0.1 M DTT	1.0
	RNase Block	1.0
	H2O	1.0
	Sum:	7 µl

- Add 7 µL master mix to each primed RT- sample (13 µL) for a final volume of 20 µL .
- Incubate at 50 °C for 02:00:00 and inactivate the reaction by heating at 70 °C for 00:15:00 .
- This is a good stopping point. Transfer samples to -20 °C .

9 Day 9: RNA-seq Library Construction Pt.2

9.1 RNase digestion

1h

- Add 1 µL DNase-free recombinant RNase A (Thermo #AM2269) and incubate at 37 °C for 01:00:00 .

Note

For the RT- samples dilute the RNase 1:5 in H2O and add 1 µl to each well.

- Perform a 1.5x SPRI bead cleanup

1. Add 150 µL of beads to the RT+ well
2. Add 30 µL of beads to the RT- well.

- Wash 2x with 80% EtOH.
- Elute the RT+ well with 145 µL H2O and transfer 20 µL of each sample to the first seven wells of a column of a new plate. Each column in this new plate corresponds to a sample.
- Elute the RT- well with 22 µL H2O and transfer 20 µL to the eighth well of the column containing the control's corresponding RT+ samples.

9.2 PCR enrichment

1. One master mix will be made for each sample and corresponding RT- reaction. The RT+ portion of the sample will be enriched in 7 individual PCRs and the RT- sample in an 8th reaction. Therefore, prepare enough master mix for ≥8.5 reactions:

Note

Reactions must be setup on ice.

	A	B
		µl per sample (RT+ and RT-)
	5x GC Buffer	85.0
	10 uM i7PCR primer	42.5
	10 mM dNTPs	12.75
	1 U/µL Kapa HiFi HotStart Polymerase	8.5
	H2O	63.75
	Sum:	212.5 µl

2. Add 25 µL master mix to each well in a column.
3. Add 5 µL of [M] 10 micromolar (µM) i5 index primer to each well in a column.
Use a different primer for each column/sample.
4. Enrich cDNAs using the following protocol:

	A	B	C
	98°C	45 s	
	98°C	15 s	5 cycles
	64.5°C	30 s	
	72°C	1 m 10 s	
	4°C	Hold	

5. Setup qPCR survey reactions using 5 µL of pre-enriched sample (from first well of a sample column) as template to determine the optimal number of PCR cycles needed for a sample. The RT- well is not sampled.

Note

Keep the pre-enriched plate covered on ice while the survey reaction runs. The polymerase is active after the hot-start and if let at room temp it will degrade the primers.

	A	B
		µl per sample
	5x GC Buffer	2.0
	10 uM i7PCR primer	1.0
	10 mM dNTPs	0.3
	1 U/µL Kapa HiFi HotStart Polymerase	0.2
	100x Sybr or 20X EvaGreen	0.09 or 0.75
	H2O	5.41 or 4.75
		9 µl

6. For each sample, add 9 µL of mix, 5 µL of sample, and 1 µL of the corresponding i5 index primer to a well of a qPCR optical plate for a final volume of 15 µL .
7. qPCR cycling conditions:

	A	B	C
	98°C	45 s	
	98°C	15 s	45 cycles
	64.5°C	30 s	
	72°C	1 m 10 s	

8. Plot signal (R) vs. cycle number. Calculate the number of additional PCR cycles needed for each sample by determining the number of cycles needed to reach 1/3 of the maximum R. If running many samples and Ct's are 'reasonably' similar, calculate the median.
9. Continue PCR on partially-amplified samples for the appropriate number (N) of cycles:

	A	B	C
	98°C	45 s	
	98°C	15 s	N cycles
	64.5°C	30 s	
	72°C	1 m 10 s	
	72°C	1 m	
	4°C	hold	

10.Perform a 0.9x (45.0 µL) SPRI bead cleanup.

Note

can pool 7 PCR wells into a deepwell plate

11. Elute the seven RT+ wells with 10 µL EB and collect 8 µL per well, pooling elutions from the same column. The final volume should be 56 µl of library per sample.

12. Elute the No-RT wells with 14 µL EB. Keep this solution in the well with the beads bound to the magnet. Draw directly from the well when preparing to run a tape in the next step.

Note

Eluting the no-RT control in this volume preserves the initial ratio of the control being 1/6 of the total captured sample (14 µl /(14 + 70 µl)). Eluting in a greater amount could dilute unwanted product.

13. Run RT+ and RT- products on the tape station with a D1000 tape.
14. **Optional,** adjust volume to 0.05% Tween for longer term storage of library.
15. Quantify DNA concentration with the Qubit system.
16. Run a MiSeq sequencing run to QC library quality before sequencing deeper.

Primer sequences

10

	A	B
	Primer name	**sequence**
	TS-2-SS-F	TAGATTGATCTAGAGCATGCACCGGACACACTCTTTCCCTACACGAC
	TS-2-SS-R	CGAAGCGGCCGGCCGAATTCGTCGAGTGACTGGAGTTCAGACGTGT
	208 TruSeq	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
	i7 PCR	CAAGCAGAAGACGGCATACGA*G*A*T
	i7 Index (#1)	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
	i5 Index ($1)	AATGATACGGCGACCACCGAGATCTACACAGCGCTAGACACTCTTTCCCTACAC*G*A*C
	RT_UMI	CAAGCAGAAGACGGCATACGAGATNNNNNNNNBGTGACTGGAGTTCAGACGTGTGCTCTTCCG*A*T*C

Protocol references

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