STARR-seq Library preparation protocol

Genome-wide library

Use 50 µg of genomic DNA *(Promega; human genomic DNA (gDNA); Cat No. G3041)*
→ yields ~ 5 µg (10%) size-selected & purified sonicated DNA for adapter ligation

Focused library (BAC)

Use 10-25µg BAC DNA *(BAC clones can be obtained from BACPAC, Invitrogen)*
In the past we have used from 10 up to 70 different BACs per library

- Pre-culture BACs in 5 ml LB+ Chloramphenicol (12.5mg/ml final conc.) individually → over-night (O.N.), 37°C
- Fill up with 50 ml LB + Chloramphenicol → grow for ~24 hours, 37°C individually
- Pool all BAC cultures
- Divide BACs in 500 ml batches and spin down, 15 min; 4000xg
- Use pellet from 500 ml pooled BAC cultures as input for 1x Qiagen Large-Construct BAC isolation
- Extract BAC DNA with Qiagen Large-Construct Kit (Cat No. 12462) according to the manufacturer's protocol
  Exception: For spinning steps use 50ml Falcon tubes at 7500xg and 50% extended spinning time
- Resuspend BACs in 300 µl TE

Sonication and size-selection of human BAC/genomic DNA (Covaris S220)

Use **5 µg** of BAC/genomic DNA in 130 µl TE per sonication (Covaris microTUBE with AFA fiber; Cat No. 520045) – in total 10-25 µg BAC DNA for a focused library and 50 µg genomic DNA for a genome-wide library

- For a target size of **1000-1500 bp**, sonicate for 15sec per 5µg sample
  - intensity parameters: 2-4-200
- For a target size of **500-750 bp** sonicate for 45sec per 5 µg sample
  - intensity parameters: 5-3-200
- Put samples on ice
- Pool all sonication samples and add the corresponding volume of 6x DNA loading dye
- Run ~60 µl sample per well (20 wells total) (do not exceed 2.5 µg/well – results in poor separation) on a 1% agarose gel at 140V for ~20-30 minutes (min) (if possible use SYBR® Safe DNA Gel Stain, Invitrogen)
- Size-select (cut) DNA between **1 kb** and **1.5 kb** (under blue light with SYBR® Safe DNA Gel Stain)
- Purify size-selected DNA from two lanes (wells) per column (~20 lanes – 10 columns total) (Gel Extraction Kit, Qiagen, Cat No. 28704). Do not use more than 400 mg agarose per column, do not heat gel slice in lysis buffer to 50ºC, 25ºC should suffice under constant agitation for 10-15 min (ensure that gel slice is completely dissolved)
- Elute in 50 µl EB, repeat elution with eluate
- Pool the elution fractions of five columns and purify again (QIAquick PCR Purification Kit, Qiagen, Cat No.28104)

- Elute in 50 µl EB, repeat elution with eluate
- Determine DNA concentration (you need 1 µg of fragmented and purified BAC/gDNA per adapter ligation reaction)
- Perform 1 adapter ligation reaction for focused libraries and 5 for genome-wide libraries

Library insert generation - Adapter ligation of sonicated BAC/genomic DNA

Perform 1 adapter ligation reaction for focused libraries and 5 for genome-wide libraries

The following protocol is based on the manual of NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®(E7645S)

Required:
1 **µg** *DNA starting material per reaction, Hairpin-adapters from NEB, USER enzyme (both contained in NEBNext® Multiplex Oligos for Illumina® (E7335L))*

1. NEBNext End Prep

1.1. Add the following components to a sterile nuclease-free tube:

Component	Volume
(green) NEBNext Ultra II End Prep Enzyme Mix	**3 µl**
(green) NEBNext Ultra II End Prep Reaction Buffer	**7 µl**
Fragmented DNA (1µg)	**50 µl**
	Total volume 60 µl

1.2. Set a 100 µl or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

1.3. Place in a thermocycler, with the heated lid set to ≥ 75°C, and run the following program:
30 minutes @ 20°C
30 minutes @ 65°C
Hold at 4°C

SAFE STOP → If necessary, samples can be stored at –20°C; however, a slight loss in yield (~20%) may be observed. We recommend continuing with adaptor ligation before stopping.

2. Adaptor Ligation

2.1. Add the following components directly to the End Prep Reaction Mixture:

● End Prep Reaction Mixture (Step 1.3)	**60 µl**
● (red) NEBNext Ultra II Ligation Master Mix*	**30 µl**
● (red) NEBNext Ligation Enhancer	**1 µl**
● (red) NEBNext Adaptor for Illumina**	**2.5 µl**
	Total volume 93.5 µl

* Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.
** The NEBNext adaptor is provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600, #E7535 and #E6609) Oligos for Illumina.

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend adding adaptor to a premix in the Adaptor Ligation Step.

2.2. Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.
(Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance).

2.3. Incubate at 20°C for 15 minutes in a thermocycler with the heated lid off.

2.4. Add 3 µl of • (red) USER™ Enzyme to the ligation mixture from Step 2.3.

Note: Steps 2.4 and 2.5 are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E7600 and #E6609) Oligos for Illumina.

2.5. Mix well and incubate at 37°C for 15 minutes with the heated lid set to ≥ 47°C.

SAFE STOP → Samples can be stored overnight at –20°C

Cleanup of adaptor-ligated DNA library with Agencourt AMPure XP beads

Since the NEBNext Ultra II Ligation Master Mix is very viscous clean up 2x with ***Agencourt AMPure XP*** *(Beckman Coulter; Cat No. A63881)*

AMPure XP bead clean-up

Note: *Keep the supernatant until confirmation that the clean-up worked*
Keep stripe on magnet at all time *(except elution)**,***
Pipette always onto the magnet opposing tube wall

- Bring beads to room temperature and mix thoroughly by vortexing before use
- Clean up **90 µl** of adaptor ligation reaction in PCR stripe
- add **1.8 vol. beads (162 µl) to 1 vol. DNA (90 µl)**, vortex, pipette up and down **20x**
- Incubate **10 min** at room temperature
- Place PCR stripe onto magnetic stand
- Incubate **10 min** on magnet until the solution is clear and all beads are collected at the wall of the tubes
- Remove all liquid
  - Wash 2x with **250 µl 80% EtOH**, incubation time **2 min**
- Air dry beads at room temperature while stripe with open lids on magnet until you see cracks (usually **2-5 min**)
- Elute by adding **100 µl RNase-free H_2O**
- Take off stripe from magnet
- Pipet up and down **25x**, close stripe, vortex and spin briefly
  - Place stripe to **37°C** in thermo cycler **for 3 min**
  - Put stripe immediately on magnetic stand, carefully tilt stand to allow beads to migrate upwards on wall, incubate for **1 min** (*or until solution is clear*)
  - Transfer eluate (= supernatant (100 µl) to new stripe

Removal of adaptor dimers using AMPure XP beads

Clean up the **entire eluate (100 µl)** from above again using AMPure XP beads.
Follow the protocol "**AMPure XP bead clean-up**" from above but add **0.8 vol. beads** to 1 vol. DNA (e.g. for 100 µl DNA use 80 µl beads)

Elute in a final volume of **20 µl EB**
Pool all 5 cleaned-up adaptor ligation reactions for genome-wide library prior to amplification

Amplification of adapter-ligated DNA library

Required:
It is critical to use *<u>KAPA HiFi HotStart ReadyMix (2X)</u>* *(Cat No. KK2601)*

Primer sequences:
(the primers below contain homology arms for cloning of the library insert to the STARR-seq screening vector by In-FusionHD cloning – see below)
in-fusion_fwd
TAGAGCATGCACCGGACACTCTTTCCCTACACGACGCTCTTCCGATCT
in-fusion_rev
GGCCGAATTCGTCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

<u>Test PCR</u>

->Aim: determination of the number of PCR cycles that result in a clear& visible product on an agarose gel (typically 6-10 cycles), based on band intensity. It is important to avoid over-amplification, which leads to concatemerization of PCR products → seen as smear on gel towards bigger sizes than expected
Perform 2 PCR reactions one with 7 & one with 9 cycles

PCR reaction

PCR reaction		
1 µl adapter-ligated DNA	Initial Denaturation:	98°C- 45 sec
2.5 µl library_cloning_fw (10 µM)		**7 and 9 cycles**
2.5 µl library_cloning_rv (10 µM)	Denaturation:	98°C- 15 sec
25 µl 2x KAPA HiFi Mix	Annealing:	65°C- 30 sec
Fill up with **H_2O** to **50 µl**	Elongation:	72°C- 45 sec
	Final elongation:	72°C- 60 sec

Gel analysis with 10 µl PCR + 2 µl 6x DNA loading dye of each test PCR
1% agarose gel, 140V, 15-30 min

<u>PCR amplification of adapter-ligated DNA library</u>

Use 1 µl of adapter-ligated DNA library per PCR reaction (50 µl); use number of cycles determined during test PCR
Perform 30 PCR reactions to amplify adapter-ligated genomic DNA library inserts (*to guarantee maximal complexity*)
For focused libraries, 4 PCR reactions are sufficient
Please see test PCR above for PCR reaction and PCR program setup

PCR purification with Agencourt AMPure XP beads

Pool 4 or 10 PCR reactions in DNA LoBind Tubes 1.5 ml (Eppendorf; Cat No. 0030108051)
Purify DNA sample with AMPure XP beads, use ***0.8 vol. beads*** *per* ***1 vol. PCR reaction***

AMPure XP bead purification

- Bring AMPure XP beads to room temperature and mix thoroughly by vortexing before use
- Add ~400 µl **(0.8 vol. beads**) of resuspended AMPure XP beads to the pooled PCR reactions
- Mix thoroughly on a vortex mixer or by pipetting up and down at least 30 times.
- Incubate for **15 min** at room temperature.
- Place tube onto magnetic stand to separate beads from supernatant
- Incubate for **10 min** then carefully remove and discard the supernatant.
- Wash **2x** with **1 ml** fresh **80% EtOH**, incubation time **2 min**
- **Air dry** beads for **~5-10 min** (until beads don't look shiny anymore and get little cracks) while the tube is on the magnetic stand with the lid open.
- Elute DNA by adding 40 / 100 µl **EB** to the beads. Mix well on a vortex mixer or by pipetting up and down.
- Incubate **5 min at 37ºC**, put the tube onto magnet, wait until the solution is clear.
- Without disturbing the bead pellet, carefully transfer the eluate to a fresh, sterile 1.5ml tube.

PCR purification (QIAquick PCR Purification Kit, Qiagen; Cat No. 74104)

This step increases cloning efficiency (In-FusionHD)

- Add **5 vol. PB buffer to 1 vol. PCR reaction** and mix.
- To bind DNA, apply the sample (max 700µl) to the QIAquick column and centrifuge for 60 sec at 18000xg
- Discard flow-through and place the QIAquick column back in the same tube.
- To wash, add **0.75 ml PE buffer** to the QIAquick column, centrifuge for 60 sec at 18000xg
- Discard flow-through and place the QIAquick column back in the same tube.
- Centrifuge for **2 min** at max. speed to remove residual PE buffer.
- Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
- To elute DNA, add **50 µl EB** (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane and centrifuge for **1 min at max. speed**.
- Re-apply eluate to column and elute again, determine DNA concentration

Library Cloning

Restriction digest and purification of STARR-seq screening vector

The purification steps are critical for cloning efficiency (In-Fusion HD)

Restriction Digest

25 µg STARR-seq screening vector *(5 µg plasmid for focused library)*
25 µl AgeI-HF
25 µl SalI-HF
50 µl CutSmart Buffer (10x)
Fill up with H_2O to 500 µl
Mix thoroughly and distribute 10x 50 µl to PCR stripes

Incubate 2h, 37ºC in PCR machine
Heat inactivate for 20 min at 65ºC

Gel extraction (QIAquick Gel Extraction Kit, Qiage, Cat No. 12362)

- Add 10 µl of 6x DNA loading dye to digested plasmid DNA
- Run **60 µl samples per lane** on a 1% agarose gel at 160V for 30 min (use **SYBR® Safe** DNA Gel Stain, Invitrogen)
- Cut out digested plasmid (~3 kb) from gel (under blue light with SYBR® Safe DNA Gel Stain)
- Purify digested plasmid DNA from two lanes per column (QIAquick Gel Extraction Kit, Qiagen). Do not use more than 400 mg agarose per column!
- Wash **2x with 700 µl PE** buffer
- Elute 2x (50 µl & 25 µl) **EB**

PCR purification (QIAquick PCR Purification Kit, Qiagen, Cat No. 74104)

- **Pool eluates** from gel extraction step and divide into two samples with equal volumes
- Clean up with 2 QIAquick columns
- Elute 2x (50 µl & 25 µl) **EB**

MinElute PCR purification (MinElute PCR Purification Kit, Qiagen, Cat No. 28006)

- **Pool eluates** from the PCR purification step and divide into two samples with equal volume
- Clean up with 2 MinElute columns
- Elute 2x (15 µl & 15 µl) **EB**

In-Fusion HD reaction (In-Fusion® HD Cloning Kit, Clontech; Cat No. 639650)

Use 2:1 molar ratio of insert (~1250 bp [including adapters]) versus plasmid (~3 kb)
Set up 40 reactions for a genome-wide library (4 reactions can be run pooled)
For a focused library set up 4 reactions in total (4 reactions can be run pooled)

In-Fusion HD Cloning Reaction
125 ng AgeI-HF/SalI-HF digested plasmid
2x molar excess PCR amplified, adapter-ligated DNA library
2 µl In-Fusion HD Enzyme Premix (5x)
Fill up with H_2O to 10 µl

→ *you can run 4 reactions per tube (40µl)*

Incubate **15 min at 50ºC** in PCR machine
Place samples on ice

Precipitation of cloned DNA library

Critical for transformation efficiency
DNA LoBind Tubes 2 ml (Eppendorf; Cat No. 0030108078)

- **Pool 4 cloning reactions** (total of ~40 µl) *(if not pooled already)*
- **Adjust** pooled cloning reactions to **250 µl with EB**
- Add **25 µl 3M NaAc pH 5.2**, vortex
- Add **2 µl Pellet Paint (Millipore)**, vortex
- Add **750 µl ice-cold (-20ºC) 100% EtOH**, vortex
- Incubate at **-20ºC 16h**
- Spin **15 min**, full speed, **4ºC**
- Vortex
- Spin again **15 min**, full speed, **4ºC**
- Carefully aspirate supernatant
- Wash **3x** with **750 µl ice-cold (-20ºC) 70% EtO**H (*mix by inverting tube or vortexing*)
- Spin **15 min**, full speed, **4ºC &** carefully aspirate supernatant
- Dry pellet for **30 sec at 37ºC**, further dry at room temperature until dry.
- Resuspend pellet of cloning reaction in **12.5 µl EB** by pipetting followed by vortexing
- Incubate for **1-3 h at -80ºC** (*increases transformation efficiency – for unknown reasons*)
- Store at -20ºC or proceed with transformation (*after freezing thaw on ice*)

Transformation of electrocompetent MegaX DH10B (Invitrogen; Cat No. C640003)

Start approximately at 4 pm
Pre-warm (1 day in advance) 12 L (4 L) LB medium at 37ºC
Pre-warm recovery medium at 37ºC
Use pre-cooled 20 µl tips (-20ºC) to pipet competent cells
Perform all steps on ice (ideally in cold room)
Pre-cool DNA LoBind Tubes 1.5 ml (Eppendorf; Cat No. 0030108051) and cuvettes (Gene Pulser Electroporation Cuvettes, 0.1 cm gap, Bio-Rad; Cat No. 1652089) on ice

- **Pool eluates** of cloning reactions (5x 12.5 µl)
- Thaw **5 tubes** (1 tube) of electrocompetent E. coli (**MegaX DH10B™** T1R, Invitrogen) **on ice** (24 transformations for genome-wide library, 3-4 transformations for BAC library)
- Distribute **2.5 µl** aliquots of pooled DNA of cloning reactions (for 24 transformations) to pre-cooled LoBind Tubes 1.5 ml
- Pipet **1 µl transformation control** (pUC19 – comes with the bacteria) to pre-cooled LoBind Tubes 1.5 ml
- Add **20 µl MegaX DH10B™ T1R** to each tube
- Pipette DNA-bacteria mix into **pre-cooled** cuvettes using **pre-cooled 20 µl tips**
- Electroporate at **2 kV, 25µF, 200 ohms** with Bio-Rad Gene PulserxCell.
- **Immediately** add **1 ml of pre-warmed** (37ºC) recovery medium (comes with MegaX DH10B™ T1R cells)
  It is critical to immediately add the pre-warmed medium (per ~ 30seconds 10-fold reduction of transformation efficiency)
- Recover transformed bacteria in **14 ml (polypropylene) round bottom tubes** (Falcon) while shaking (>300 rpm) for **1 h at 37ºC**
- **Pool** all transformations *(~22-23 ml*)
- Make a **dilution series** of pooled bacteria culture from transformation reaction and transformation control to determine efficiency:
- 1:10 100 µl bacteria culture + 900 µl LB medium
- 1:50 200 µl from 1:10 dilution + 800 µl LB medium
- 1:500 100 µl from 1:50 dilution + 900 µl LB medium
- 1:5000 100 µl from 1:500 dilution + 900 µl LB medium
- Plate 100 µl of 1:50, 1:500 and 1:5000 dilutions on Ampicillin selection plates (100 µg/ml)
- Add **equal volumes** (~1.8 ml) of bacteria culture from pooled transformation reactions to 2L of pre-warmed (37ºC) LB medium with Ampicillin (100 µg/ml) in 5 L Erlenmeyer flasks
- Incubate overnight (**~13 h**) while shaking (200 rpm) at 37ºC
- Measure OD_{600} (**OD_{600}** should be between **2 - 2.6**)
- **Spin** down bacteria culture in 1L bottles, **30 min (45 min), 4200 rpm, 4ºC**
- Decant supernatant (leave small volume of medium)
- Resuspend bacteria pellets in remaining medium by vortexing
- **Pool** resuspended pellets **into one bottle**
- Use 10-15 ml of LB medium (per 4 bottles) to clean out remaining pellet remains, unite everything with resuspended pellets and vortex to obtain a homogenous bacteria

suspension

- **Distribute** bacteria suspension evenly **into 8 (or up to 10) 50 ml falcon tubes** (before determining weight of empty tubes)
- Spin **15 min, 6000xg at 4ºC**
- Aspirate supernatant
- Determine weight of bacteria pellets in falcon tubes
- Store pellets at -20ºC

Purification of cloned DNA library (Plasmid Plus Giga Kit, Qiagen, Cat No. 12991)

For Electroporation of cells using the MaxCyte STX transfection system it is critical to have the DNA (library) suspended in H_2O at a high concentration (> 1 µg/µl)

- Use one pellet (max. 7.5 g) and follow the protocol for the Plasmid Plus Giga Kit
- **Elute with 1 ml H_2O**, measure concentration
- Elute again with 0.5 ml **H_2O**, if concentration is > 2 µg/µl add to the 1st elution, if <2 µg discard it and continue only with the 1st elution.