STARR-seq Screening protocol

Alexander Stark (Alexander.Stark@imp.ac.at)
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Felix Muerdter
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Łukasz M. Boryń
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Ashley R. Woodfin
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Christoph Neumayr
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Martina Rath
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Muhammad A. Zabidi
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Michaela Pagani
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Vanja Haberle
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Tomáš Kazmar
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Rui R. Catarino
Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Katharina Schernhuber

Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

Cosmas D. Arnold

Research Institute of Molecular Pathology (IMP), Vienna Biocenter (VBC), Campus-Vienna-Biocenter 1, 1030 Vienna, Austria

---

Method Article

Keywords: enhancer, reporter assay, luciferase, starr-seq, mpra

Posted Date: December 11th, 2017

DOI: https://doi.org/10.1038/protex.2017.148

Abstract

Self-transcribing active regulatory region sequencing \(STARR-seq) is a massively parallel reporter assay to identify transcriptional enhancers directly based on their activity in entire genomes for millions of candidates from arbitrary sources of DNA and to assess their activity quantitatively \(Arnold et al., Science 2013). Enhancer activity is directly linked to the underlying DNA sequence and measured as presence of the resulting reporter transcripts among cellular RNA by deep sequencing. This protocol describes the screening of STARR-seq libraries in human cells.

Reagents

Recommended: For BAC screens : T-225 flasks \(Thermo Scientific; Cat No. 159934) For genome-wide screens: Square plates \(Thermo Scientific; Cat No. 166508) Required: C16 inhibitor targeting PKR \(Sigma-aldrich; Cat No. I9785-5MG) BX-795 inhibitor targeting TBK1/IKK \(Sigma-aldrich; Cat No. SML0694-5MG) 1x PBS \(Autoclaved), 1x Trypsin \(Gibco; Cat No. 25300 054) DMEM \(Gibco; cat. no. 52100-047) + 10% heat-inactivated FBS \(Sigma; Cat No. F7524) + 2 mM L-glutamine \(Sigma; Cat No. G7513) Electroporation device: MaxCyte STX scalable transfection system Electroporation buffer \(MaxCyte; Cat No. EPB1) Clinical Processing Assembly, OC-100 100-µL \(MaxCyte; Cat No. GOC1) Clinical Processing Assembly, OC-400 400-µL \(MaxCyte; Cat No. GOC4)

Procedure

Focused \(BAC) screen: Grow cells and split them 24 h before transfection to reach $8x10^7$ cells on electroporation day; \(e.g. HeLa S3, 2 square plates, $4x10^7$ cells/square plate) Genome-wide screen: Grow cells and split them 24 h before transfection to reach $8x10^8$ cells on electroporation day; \(e.g. HeLa S3 , 25 square plates , $4x10^7$ cells/square plate) Prepare before transfection: 1x / 4x T225 flasks at 37°C, 2 / 7 square plates at 37°C, and 1mM stocks of the C16 and BX-795 inhibitors at -20°C **1. Electroporation of mammalian cells using the MaxCyte STX transfection system** • Remove medium from cells completely • Wash cells carefully with 10 ml PBS, remove PBS completely • Add 1x Trypsin to the cells to cover the plate/flask completely \(Square plate: 8ml Trypsin) • Incubate cells on 37°C / 4-5 min \(or until cells completely detach) • Add 12 ml medium to the cells, resuspend by pipetting up and down thoroughly • Collect cells in a T-225 flask • Count cells and use $8x10^7$ / $8x10^8$ cells for the transfection \(it is possible that the amount of medium & cells for a gw-screen will not fit in 1x T225 flask, use 2x T225 flasks and measure each flask separate • Spin the cells down \(in 50 ml Falcon tubes) at 125xg for 5 min • Remove the medium, resuspend pellets in 5 ml Electroporation buffer and pool all pellets in a 50 ml falcon tube \(do not exceed ~40 ml if you have many pellets, reuse cell suspension of one pellet to resuspend the next if necessary) • Spin the cells down 125xg, 5 min • Remove medium and add 500 µl / 5 ml Electroporation buffer to the cells \(after resuspension of the cell pellet the volume is around 650 µl/ 7 ml) • Resuspend the cells and add appropriate amount of library to the cells \(library concentration should be > 1.0 µg/ µl) • Use 80 µg library per $4x10^7$ cells in 400 µl Electroporation buffer per OC-400 cuvette • for a focused

screen $8x10^7$ cells are needed, \(2x OC-400 cuvettes • for a genome-wide screen $8x10^8$ cells are needed, \(20x OC-400 cuvettes) • Measure volume and fill resuspension up to 800 µl/ 8 ml total with Electroporation buffer • Add 400 µl cell suspension/ DNA mix per OC-400 cuvette and electroporate using the cee-type specific pre-set protocols or optimization protocol \(if no pre-set protocol is available for your cell line use the best fitting optimization protocol for your cell line) MaxCyte STX scalable transfection system • Put 2x / 10x EPOs into 1x pre-warmed T225 flask, without adding medium • Put cells into the incubator, 37°C for 30min • Repeat with the additional 10 EPOs ands put them T225 flasks, inc. at 37°C for 30min • After 30 min add 100ml/ 350ml medium without antibiotics on the cells • Start counting the 6 hours incubation time after medium has been added to the cells \(count 6 h until cells lysis during total RNA extraction; see below) • For cells with Interferon \(INF) response add 100 µl or 350 µl of a 1 mM stock of the C16 and BX-795 inhibitors to the cells \(final concentration 1 µM/ inhibitor). • Don't pipette cells up and down BUT close flasks and shake them mix with inhibitors • Take out 2x / 7x 50ml and put cells into the pre-warmed 2 / 7 square plates or flasks **2. Harvesting of mammalian cells 6 hours post electroporation** Note: Harvesting of adherent cells, especially for a genome-wide screen can take up to 1.5h, start therefore early to stay in the 6h time window, ideally work together with a partner. • Harvest the medium of the cells \(20% of cells are still not fully attached to the plates), add 10 ml PBS, harvest PBS, add 8 ml of 1x Trypsin, put cells in incubator 37°C wait 4-5 min, then add 12 ml of medium \(to save some medium: Re-use the pre-harvested medium to inactivate the trypsin), resuspend thoroughly and harvest • Spin the cells down \(in 50ml Falcon tubes) at 125xg for 5 min • Remove medium • Wash cells once with 10 ml/ 40 ml 1x PBS • Spin the cells down \(in 50 ml Falcon tubes) at 125xg for 5 min • Remove 1x PBS but leave a rest of the 1x PBS \(0.5 ml/ 1 ml) to cover the cells in the 50 ml falcon • Resuspend the cells in the 1x PBS by softly flicking on the side of the 50 ml falcon **3. Total RNA Purification \(RNeasy Maxi Kit, Qiagen; Cat No. 75162)** Work for this step in in a RNAse free fume-hood to avoid β-Mercaptoethanol exposure, use a Tissue Ruptor \(Qiagen; Cat No. 9001271), RNase Zap \(Ambion; Cat No. AM9780), clean all equipment with RNAse Zap. Prepare fresh: • 15 ml RLT \(in the RNeasy Maxi Kit) buffer • 15 ml 70% ethanol per cell pellet \($8x10^7$- $5x10^8$) • add 150 µl β-Mercaptoethanol \(β-ME) to 15 ml RLT buffer • add 4 volumes of 100% ethanol to RPE buffer • prepare 70% ethanol \(dilute with DEPC-treated sterile MonoQ water) **Purify RNA:** • add 15 ml RLT \(+β-ME) buffer while vortexing to the resuspended pellet • disrupt and homogenize the cells with TissueRuptor for 4.5 min per cell pellet • add 15 ml 70% ethanol to the lysate, shake/mix vigorously \(20") • transfer to RNeasy Maxi column \(max. volume 15ml) centrifuge for 5 min at >3200xg, RT, discard the flow-through • repeat with the remaining 15ml lysate/EtOH mix • add 15 ml RW1 buffer, centrifuge for 5 min at > 3200xg, 25°C, discard the flow-through • add 10 ml RPE buffer, centrifuge for 2 min at > 3200xg, 25°C, discard the flow-through • add 10 ml RPE buffer, centrifuge for 10 min \(to dry the membrane) at > 3200xg, 25°C, discard the flow-through • take new tubes and elute 3x in RNase free H2O: 1st in 1.2 ml, 2nd in 1 ml, 3rd in 0.5 ml • incubate for 2 min and centrifuge for 5 min If concentration is expected to be low e.g for focused screens, elute in 0.5 ml steps only If concentration after 3rd elution > 300 ng: elute further in 0.5 ml steps. • if using more than one column per sample purification \(typically for genome-wide screens), pool same elution fractions to obtain pools of the 1st, 2nd and 3rd elution steps • measure RNA concentration of pooled elution fractions • mix 1st, 2nd and 3rd elution fractions to obtain a pool with ~750 ng/µl \(that's

the max. concentration you can use in the next step) • Samples can be stored at -80°C • safe 10 µl for gel analysis to determine integrity of RNA \(check for degradation) **4. Oligo-dT mRNA Isolation \(Dynabeads Oligo\(dT)25, Invitrogen; Cat No. 61005)** Prepare before starting: • Binding buffer \(2.5x starting volume of beads, 20mM TrisHCL pH7.5, 1M LiCl, 2mM EDTA) • Washing buffer \(2x starting volume of beads, 10mM TrisHCL pH7.5, 0.15M LiCl, 1mM EDTA) Warm all buffers up to room temperature \(RT) Prepare RNA for binding to Dynabeads, he maximum concentration of RNA is 750 ng/µl: For 15 ml tubes \(polystyrene tubes Cat No. 05-527-90) • heat total RNA for 12 min at 65°C, place on ice immediately for 5 min, wait for 1 min at room temperature For 1.5 ml DNA LoBind Tubes \(Eppendorf; Cat No. 0030108051) • heat total RNA for 7 min at 65°C and incubate for 3 min on ice; 1 min, room temperature **Oligo-dT selection of mRNA with Dynabeads** Use 2x vol. of Oligo\(dT)25 beads for 1x vol. of total RNA solution, do not vortex as long as RNA is bound to beads\! • resuspend the Dynabeads Oligo\(dT)25 beads thoroughly by vortexing to obtain a uniform brown suspension, distribute beads to appropriate tube & place the tube on a magnet • remove Storage Buffer completely from beads • wash beads 2x with 2x Binding Buffer \(same volume like beads starting volume): after adding buffer, vortex and incubate beads on a magnetic separator for 1.5 min \(until solution is clear) • resuspend the beads in ½ volume \(referring to starting volume of beads) of 2x Binding Buffer • add 1 vol. total RNA solution – to 1 vol. resuspended beads > mix gently \(pipet up and down) • incubate on a rolling shaker for 10 min at RT • place the tube on the magnet for 2 min and completely remove supernatant • carefully wash the beads 2x with Washing Buffer B \(use same volume as beads starting volume): after adding buffer mix gently by inverting the tube \(no vortex) and incubate for 1.5 min on the magnetic separator • before elution spin tube very shortly and remove the remaining buffer on the magnet • elute by adding 50 µl + 40 µl 10 mM Tris-HCl per 1 ml beads \(starting volume); vortex • place beads on 80°C heat block for 3 min, 750rpm; transfer tubes immediately to the magnet and incubate for 1 min, transfer supernatant to a new RNase-free tube • pool all 1st and 2nd elutions from same sample • measure RNA concentration of final pool • safe 10 µl of pooled elution fractions for gel analysis • based on determined RNA concentration load appropriate amounts on gel \(do not load more than 1 µg of RNA per lane) **5. Turbo DNaseI Digest** Turbo DNase I \(Ambion; Cat No. AM2238) is sensitive to mechanical stress\! Do not vortex. Process the entire mRNA, scale up number of reactions accordingly. For one reaction: 88 µl mRNA \(max. 200ng/µl) 10 µl Turbo DNase Buffer 2.4 µl Turbo DNase I • make Master Mix • invert gently • distribute 100µl MM to PCR-stripes • incubate at 37°C for 30 min at 37°C, 4°C for ∞ **6. Clean up with Qiagen RNeasy MinElute clean up kit \(Cat No. 28204)** Prepare 80% EtOH \(with DEPC water) and 100% EtOH, DNA LoBind Tubes 2 ml \(Eppendorf; Cat No. 0030108078) • pool 2 DNase rxns \(or fill up with water to 200 µl) in 2ml LoBind tubes • add 700 µl RLT buffer, vortex • add 500 µl 100% EtOH, vortex • transfer to RNeasy MinElute spin column \(max. vol. 750µl) • centrifuge at 12000xg, 30 sec in a table top centrifuge and discard the flow-through • place RNeasy MinElute spin column in a new 2 ml collection tube \(provided in kit) • add 500 µl RPE buffer, centrifuge at 12000xg, 30 sec and discard the flow-through • add 500 µl 80% EtOH, centrifuge at 12000xg, 2 min and discard the flow-through • place RNeasy MinElute spin column in a new 2 ml collection tube \(provided in kit) • to dry the membrane, centrifuge 5 min at full speed with open lid • elute in 20 µl + 10 µl RNase-free H2O • incubate for 1 min and centrifuge for 3 min at full speed • pool eluates from all columns • measure RNA concentration of final pool • measure volume of

final pool • safe 5 µl of final pool for gel analysis **7. Reverse Transcription \(SuperScriptIII; Invitrogen; Cat No. 18080093)** Use max. 4-5 µg RNA per RT rxn , perform 1 negative control \(RTminus/ RT-) \(add RNase free H2O, instead of SSIII), if RNA concentration is low \(5-10 µg RNA in total), do at least 5 rxn\! X µl RNA \(max. conc. 4-5ug) 1 µl dNTP 1 µl GSP \(2 µM) \(1:50; gene specific primer; CTCATCAATGTATCTTATCATGTCTG) fill up to 13 µl with RNase-free H2O • make Master Mix • distribute 65µl MM to PCR-stripes \(5 reactions/tube) • 65°C for 5 min, put on ice for 1 min Prepare next \(per rxn): 4 µl 5x First-Strand buffer 1 µl DTT \(0.1 M) 1 µl RNaseOUT 1 µl SSIII \(H2O for -RT control) • add 35µl of this enzyme mix to the previous 65 µl rxn \(5 rxns/tube: final vol.: 100µl) • incubate at 50°C for 1h, 70°C for 15 min , 4°C for ∞ Optional: samples can be stores at -20°C **8. RNaseA treatment** • add 1 µl RNaseA \(10mg/ml; Fermentas; Cat No. EN0531) per 100 µl reaction \(= 5 rxns) • add 0.2 µl RNaseA per RT- rxn \(= 20 µl) • incubate for 1 h at 37°C Optional: samples can be stores at -20°C **9. cDNA purification with AMPure XP beads \(Beckman; Cat No. A63882)** • pool all cDNA samples, mix thoroughly and distribute up to 300 µl \(= 15 RT rxn; keep RTminus separate) to LoBind 1.5ml tubes • Mix/vortex beads thoroughly before use • add 1.8 vol. beads to 1 vol. cDNA \(36 µl for –RT), vortex, pipette up and down 20x • incubate 15 min at room temperature • place onto magnet • incubate 15 min on a magnetic stand • the beads should be collected to the wall of the tube and the solution should be clear • remove all liquid • wash 2x with 1 ml 80% EtOH, incubation time 2 min \(beads have to be covered) • dry beads at room temperature for 5-10 min \(keep the tubes with open lids on the magnet) • elute by adding 20 µl EB per rxn \(15 rxn: elute in 300 µl) • take tubes off the magnet • pipet up and down 25x • place tubes on 37°C heat block, shake at 300 rpm for 3 min, • put tubes immediately on magnetic stand, carefully tilt stand to allow beads to migrate upwards on wall, incubate for 1 min • transfer supernatant \(=sample) to new tube • pool all cleaned cDNA samples \(except RTminus) **10. Junction PCR \(jPCR)** Note: Number of jPCR = number of RT reactions, jPCR primers depend on intron used \(chimeric etc.), • = phosphorothioate bond jPCR mix: 20 µl cDNA \(do 1 jPCR per RT rxn) 25 µl KAPA 2xHiFi 2.5 µl junction fwd \(10 µM) \(TCGTGAGGCACTGGGCAG•G•T•G•T•C) 2.5 µl junction rev \(10 µM) \(CTTATCATGTCTGCTCGA•A•G•C) • fill up with H2O to 50 µl PCR program: 98°C- 45 sec; \(98°C- 15 sec, 65°C- 30 sec, 72°C- 70 sec) 15 cycles; 72°C- 60sec **11. jPCR purification with AMPurexP beads** • pool all reactions \(up to 12 jPCR rxn = 600 µl), keep –RT separate • Measure volume of pooled reactions \(always lower than added volumes of individual reactions) • Mix/vortex beads thoroughly before use • add 0.8 vol. beads to 1 vol. cDNA \(40 µl for –RT), vortex, pipette up and down 20x • incubate 15 min at room temperature and another 15 min on a magnetic separator • the beads should be collected to the wall of the tube and the solution should be clear • remove all liquid • wash 2x with 1 ml 80% EtOH, incubation time 2 min \(beads have to be covered) • dry beads at room temperature for 5-10 min \(keep the tubes with open lids on the magnet) • take tubes off magnets • elute in 20 µl H20 per rxn \(25rxn, elute in 500 µl) • pipet up and down 25x • place beads on 37°C heat block, shake at 300 rpm for 3 min, • put beads immediately on magnetic stand, carefully tilt stand to allow beads to migrate upwards on wall, incubate for 1 min • transfer supernatant \(=sample) to new tube • pool all cleaned jPCR samples \(except RT-) **12. Sequencing ready PCR** First do a Test PCR \(Figure 1) to test how many cycles are necessary, you need to see a clear band corresponding to the length of the library input \(5, 9 cycles). PCR mix: 20 µl jPCR \(from pool) 2.5 µl PE1.0 \(10 µM) 2.5 µl MP2.0 \(10 µM) 25 µl KAPA 2x HiFi Mix PCR

program: 98°C- 45 sec; \(98°C- 15 sec, 65°C- 30 sec, 72°C- 45 sec) 5,9 cycles; 72°C- 60sec Gel analysis: 1% agarose gel, 140V, 15 min load 10 µl of each test PCR + 2 µl of 6x DNA loading dye Determine how many cycles to use based on band intensity after 5 and 9 cycles. For example, if you see a faint band at 5 cycles and overamplification after 9 cycles, we recommend 5 cycles. If 5 cycles already result in an overamplification, reduce the amount of template \(divide by 2 per cycle less, for example 5 µl template and 5 cycles if 3 cycles would suffice. Do not reduce number of cycles for the junction PCR to compensate\!) **Sequencing ready PCR:** PCR mix: 20 µl jPCR \(purified) 2.5 µl PE1.0 \(10 µM) 2.5 µl TruSeq IDX \(10 µM) \(appropriate barcodes, see sequences on next page) 25 µl KAPA 2x HiFi Mix PCR program and number of cycles: see Test PCR Number of reactions: 2 for focused and 20 for genome-wide screen PE1.0: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT MP2.0: CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT **13. SeqReady PCR purification with SPRIselect beads \(Beckman; Cat No. B23318)** It is critical to use the exact beads to PCR ratio – using less than 0.5 leads to loss of sample. • pool 2 rxn or 10 rxn \(pipette 45 µl out of each PCR reaction to get the exact volume) • Mix/vortex beads thoroughly before use • add 0.5 vol. beads to 1 vol. DNA, vortex, pipette up and down 20x • incubate 10 min at room temperature and another 5 min on magnet in LoBind 1.5ml tube • the beads should be collected to the wall of the tube and the solution should be clear • remove all liquid • keep tubes on magnets all the time \(except elution), pipette on opposite tube wall • wash 2x with 1 ml 80% EtOH, incubation time 2 min • dry beads at room temperature for 2-5 min \(keep the tubes with open lids on the magnet) • elute by adding 10 µl RNase free H2O \(no EB) per rxn \(2 rxn: 20 µl, 10 rxn: 100 µl) • take off tubes from magnet • pipet up and down 25x • place tubes to 37°C on heat block for 3 min, • put tubes immediately on magnetic stand, carefully tilt stand to allow beads to migrate upwards on wall, incubate for 1 min • transfer supernatant \(=sample) to new tube • pool all cleaned seq ready PCR samples \(except RTminus)

Figures

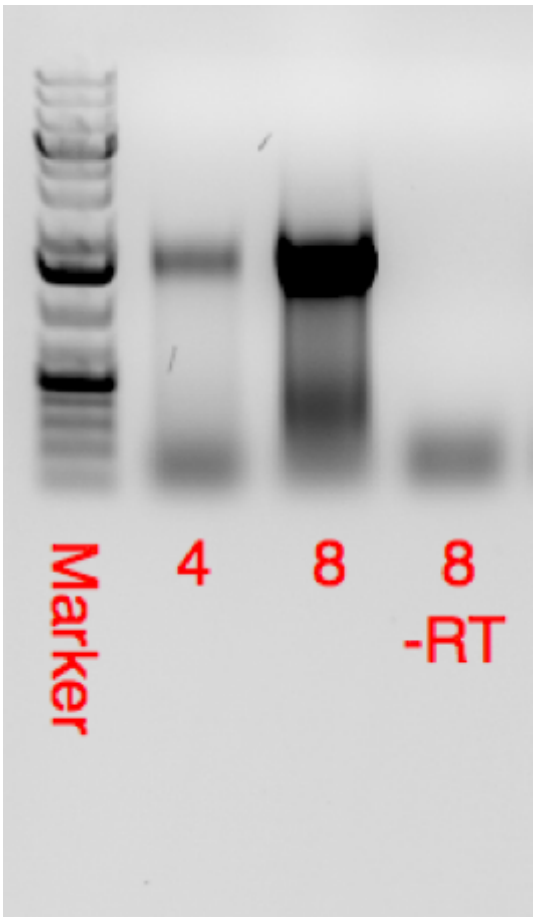

Figure 1

Test PCR to determine appropriate cycle number Loading scheme: 1: Fermentas GeneRuler 1kb plus, 2: 10µl of a test PCR (4 repeats, 5 cycles total), 3: 10µl of a test PCR (8 repeats, 9 cycles total), 4: 10µl of the -RT control (8 repeats, 9 cycles total).