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RoCK and ROI: bead modification, library generation and sequencing protocol

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Abstract

Various tools have been developed to reliably identify, trace and analyze single cells in complex tissues. In recent years, these technologies have been combined with transcriptomic profiling approaches to explore molecular mechanisms that drive development, health, and disease. A remaining challenge is that important information relevant for understanding the biology of cells or tissues, such as lowly expressed transcripts, sequence variations or exon junctions, remains undetected. We developed an scRNAseq workflow, **RoCK and ROI** (Robust Capture of Key transcripts and Region Of Interest), that tackles these limitations. **RoCKseq** uses targeted capture to enrich for key transcripts, thereby enhancing the detection, identification and tracking of cell types in scRNAseq experiments. **ROIseq** directs a subset of reads to a specific region of interest via selective priming. This allows specific sequence information to be retrieved for mRNAs of interest, enabling, for example, the inspection of sequence variations. Importantly, the targeted information obtained with RoCK and ROI is recorded together with standard transcriptome readouts. To analyze the multimodal information provided by RoCK and ROI, we developed a novel pipeline. The entire workflow increases the information obtained for lowly expressed genes and enables the detection of individual sequence variations and the exploration of the biological relevance and consequences of the respective variation for the cells expressing it.

This protocol covers the following steps:

- Design of RoCKseq capture sequences and ROIseq primers
- RoCKseq bead modification on BD Rhapsody beads
- RoCK and ROI library generation
- Sequencing of RoCK and ROI libraries

Guidelines

IMPORTANT: This protocol refers to “Enhanced Cell Capture Beads V2” (Part Number: 700034960). For “Enhanced Cell Capture Beads V3” (Part number 91-1294), the sequence of the splint is:

5’-NNNNNNNNNNNNNNNNNNNNNNNNTATAATCACGACTCCAC-3’

Materials

RoCKseq bead modification:

Buffers and reagents:

- T4 polymerase (Thermo scientific EP0061)
- Lambda exonuclease (NEB M0262)
- Tris, pH 8.0: Invitrogen (ThermoFisher AM9856)
- EDTA, pH 8.0: Invitrogen (ThermoFisher AM9261)
- Tween20 (Thermo scientific 13464259)
- dNTPs (10 mM)
- ddH_2O
- BD Rhapsody barcoded beads (“Enhanced Cell Capture Beads V2”, part Number 700034960)
- Splint(s) (100 µM)
- polyA oligo of 18 nucleotides (100 µM)

- TE/TW buffer: 500 µl Tris, 100 µl EDTA, 10 µl Tween20, up to 50 mL with ddH2O
- Water buffer: 10 µl Tween20, up to 50 mL with ddH2O

Consumables:

- 1.5 mL DNA LoBind tubes (Eppendorf 0030108418)
- LoBind pipette tips (multiple vendors)
- 50 mL Falcon tubes

Equipment:

- Magnetic stand for 1.5 mL tubes (multiple vendors)
- 2x thermomixers (Eppendorf)
- MACSmix tube rotator Miltenyi

Fluorescent assay:

Buffers and reagents:

- BD Rhapsody Lysis buffer (part number 650000064 of Cartridge Reagent Kit)
- BD Rhapsody DTT (part number 650000063 of Cartridge Reagent Kit)
- BD Rhapsody beads (“Enhanced Cell Capture Beads V2”, part Number 700034960)
- Tris, pH 8.0: Invitrogen (ThermoFisher AM9856)
- EDTA, pH 8.0: Invitrogen (ThermoFisher AM9261)
- Tween20: Thermo scientific 13464259

- TE/TW buffer: 500 µl Tris, 100 µl EDTA, 10 µl Tween20, up to 50 mL with ddH2O
- Water buffer: 10 µl Tween20, up to 50 mL with ddH2O

Consumables:

- 1.5 mL DNA LoBind tubes (Eppendorf 0030108418)
- LoBind pipette tips
- Falcon 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap (Corning 352235)
- Aluminum foil

Equipment:

- Magnetic stand for 1.5 mL Eppendorf tubes
- Thermomixer

Library generation:

- BD Rhapsody™ Enhanced Cartridge Reagent Kit: BD 664887
- BD Rhapsody™ Cartridge Kit: BD 633733
- BD Rhapsody™ cDNA Kit: BD 633773
- BD Rhapsody™ WTA Amplification Kit: BD 633801

List of primers:

	Name of primer	Sequence (5' to 3')	Modification	Purification	Scale	Dilution	Concentration
	TSO protective oligo	CATACCTACTACGCATA	5' phosph	HPLC	0.2 µmol	ddH2O	100 µM
	T primer	ACAGGAAACTCATGGTGCGT	-	HPLC	0.2 µmol	DNA resuspension buffer	100 µM
	Sequencing primer	ACACTCTTTCCCTACACACAGGAAACTCATGGTGCGT	-	HPLC	0.2 µmol	ddH2O	100 µM
	T primer + adapter	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACACAGGAAACTCATGGTGCGT	-	IEX - HPLC	0.2 µmol	DNA resuspension buffer	100 µM
	PolyA protective oligo	AAAAAAAAAAAAAAAAA	5' phosph	HPLC	0.2 µmol	ddH2O	100 µM

DNA resuspension buffer: DNA Supension buffer from Teknova (T0221)

List of fluorescent oligos:

	Name of fluorescent oligo	Sequence (5' to 3')	Modification	Purification	Scale	Dilution	Concentration
	polyA fluo oligo	AAAAAAAAAAAAAAAAAAA	5' Atto647N	HPLC	0.2 µmol	ddH2O	100 µM
	TSO fluo oligo	CATACCTACTACGCATA	5' Atto647N	HPLC	0.2 µmol	ddH2O	100 µM

Before start

Important points to keep into consideration during RoCKseq bead modification

- LoBind DNA tubes and pipette tips guarantee low bead loss during modification, which otherwise get stuck on walls of pipette tips and tube
- Beads should be kept on ice whenever possible
- Bead modification should be performed in a clean, RNAse-free hood
- Enzymes should be kept at -20°C as long as possible and buffers and splints should be placed on ice after thawing
- If multiple samples are processed in parallel, only wash up to four samples at a time to prevent incubation on the magnetic stand for too long
- Try limiting (i.e. restrict to 1 minute) the time the beads are exposed to the magnetic stand
- Avoid the drying out of the beads after washing
- To minimise bead loss during modification: consistently use LoBind DNA Eppendorf tubes and LoBind pipette tips and wait for all the beads to be gathered at the magnet of the magnetic stand before exchanging buffers. During washes ensure that all liquid is expelled from the tip as to minimize bead loss

Important points to keep into consideration during the Fluorescent assay

- After addition of the lysis buffer keep beads at room temperature. Do not place back on ice. This may lead to higher fluorescent background signal in the negative control
- The fluorescent probes and the beads with the fluorescent probe should be kept in the dark whenever possible

Design of capture sequences

1 Before proceeding with the bead modification step, splints and fluorescent oligo need be designed and ordered

2 **Points to keep into consideration when designing splints:**

- The GC content of splints should be in the range of 40-60%. Higher GC content may impair reverse transcription (i.e. first strand synthesis). Also consecutive GC stretches of more than 4 bases should be avoided. Similarly a low GC content and longer stretches of A should be avoided in order to prevent dT-based capture of the target transcript
- GC content upstream of splint: if the GC content of the transcript of interest upstream of the splint is too high (more than 5 consecutive G or Cs), this may impair reverse transcription (i.e. first strand synthesis)
- Length of the splint: 24 nucleotides
- Place the capture whenever possible into the CDS of the transcript of interest: the 3'- and 5' UTRs are less conserved and thus more prone to accumulate nucleotide polymorphisms that will hamper targeted capture. For long non-coding RNAs we suggest capturing the transcript in a conserved region whenever possible. Sequencing the locus in the strain used is recommended.
- Vicinity to ROIseq primer: when performing RoCK and ROI, the splint should be chosen not more than 300 - 400 bp downstream of the ROIseq primer. This accounts for the sequence on the bead (primer, barcode, UMI, TSO). Please note adaptors for sequencing add to the final product size as well.
- G or a C at the 5' end of the splint (and thus 3' end of the capture) favor reverse transcription.
- The capture should not be overlapping with known splice junctions: this may be an issue if unknown splice variants are present (i.e. intron retention)

3 **Splint sequences**

IMPORTANT: all splints are 5' phosphorylated

The sequence of the splint for the modification of TSO oligos on BD Rhapsody "Enhanced Cell Capture Beads V2" is as follows:

5’ -24 or 25 nt coding sequence followed by a constant sequence-3’:

5’-NNNNNNNNNNNNNNNNNNNNNNNNNCATACCTACTACGCATA-3’

where the CATACCTACTACGCATA is the reverse complement of the TSO sequence on the beads.

The polyA protective oligo used on the barcoded beads is 18 nucleotides in length:

5’-AAAAAAAAAAAAAAAAAA-3’

The oligos should be ordered in 0.2 µmol scale, HPLC grade, with **5’ phosphorylation**. Before use, resuspend the oligos in ddH2O to generate a 100 µM stock solution.

IMPORTANT:

- To modify RoCKseq beads with multiple capture sequences, mix the splints in the desired ratio. For example, to modify RoCKseq beads with the same amount of three splints (33% each), pipette 5 µL of each splint and mix with 15 µL of 100 µM polyA oligo
- The modification of RoCKseq beads can be titrated to achieve different amounts of modification on TSO oligos. The titration is achieved by mixing the splint(s) with the protective TSO oligo. This oligo is also 5' phosphorylated. For example, to achieve a 50% of RoCKseq modification, a mix of 7.5 µL of splint(s) and 7.5 µL of protective TSO oligo is generated and mixed with 15 µL of 100 µM polyA oligo

Design of fluorescent oligos

4 To design the fluorescent oligos, take the **first 20 nucleotides from the 5’ end of the splint**.

The fluorescent oligos should be ordered in HPLC grade and in 0.2 µmol scale with a **5’ Atto647N** modification and diluted in ddH_2O to generate a 100 µM stock solution.

Note

The same fluorescent moiety is used for all fluorescent oliogs as the BD Rhapsody beads are autofluorescent in other channels

RoCKseq bead modification protocol for splint testing

5 **IMPORTANT: the protocol described below refers to the modification of a full vial of BD Rhapsody barcoded beads. Alternatively, to test the efficacy of the bead modification with new capture sequences, the protocol can be adapted to modify a small aliquot of beads.**

Instead of 2 mL of BD Rhapsody barcoded beads per sample, 20 µL of beads can be used. The same protocol can be used with the following changes:

Step 9: T4 polymerase mix: 40 µL buffer, 20 µL 10 mM dNTPs, 136 µL ddH2O
Step 28: 1 µL of polyA – splint mix
Step 31: 1 µL T4 polymerase enzyme
Step 34: lambda exonuclease mix: 15 µL reaction buffer, 132 µL ddH_2O
Step 40: 3 µL lambda exonuclease enzyme

The fluorescent assay protocol can be used as described below, with all 20 µL of modified beads being used as input.

Step 1 modification of full vial of RoCKseq beads: preparation of reagents 15m

6 Thaw lambda exonuclease buffer, T4 polymerase buffer, 100 µM splint(s), polyA oligo and 10 mM dNTPs at room temperature and place On ice

7 Preheat two thermomixers to 75 °C and to 37 °C, respectively

8 Prepare TE/TW and Water buffers in 50 mL Falcons and place On ice

- TE/TW buffer: 500 µL Tris, 100 µL EDTA, 10 µL Tween20, up to 50 mL with ddH_2O
- Water buffer: 10 µL Tween20, up to 50 mL with ddH_2O

Note

- Keep TE/TW and Water buffers on ice as much as possible as increased temperature may impact the modification rate on the beads
- TE/ TW and Water buffers should be prepared freshly for each bead modification

Note

Tween20 is viscous, the pipette tip may need to be cut to increase the size of the opening

9 **Preparation of T4 polymerase mix**: Prepare four 1.5 mL DNA LoBind tubes. Pipette into each tube: 260 µL T4 polymerase buffer, 130 µL 10 mM dNTPs, 857 µL ddH_2O and place On ice

10 **Preparation of splint mix**: Pipette 15 µL of 100 µM polyA oligo and 15 µL of 100 µM splint into new 1.5 mL DNA LoBind tube. If a mix of splints is used, pipette 15 µL of 100 µM polyA oligo and 15 µL of mix of splints (see Step 3)

Note

IMPORTANT: The addition of the polyA oligo is critical, as it protects the dT oligos on the beads from degradation. Omission of the polyA oligo leads to a lower number of genes and UMIs detected in scRNAseq experiments

11 Incubate splint mix in thermomixer at 75 °C for 00:05:00 without shaking and place On ice

12 **Preparation of beads**: Resuspend the beads by gently pipetting up and down with a 1 mL pipette set to 500 µL being careful not to lose any supernatant. Immediately transfer the 2 mL of barcoded beads provided by the manufacturer by pipetting 500 µL of BD Rhapsody barcoded beads into four new 1.5 mL DNA LoBind tubes and place On ice. After the transfer to each tube resuspend the remaining beads by pipetting up and down to allow for a similar amount of beads being transferred per replicate

Note

Barcoded beads should be kept on ice as much as possible to avoid degradation of the DNA oligos on the beads

13 Proceed immediately to “Washing BD Rhapsody beads”

Step 2 modification of full vial of RoCKseq beads: washing BD Rhapsody beads

5m

14 Place the four 1.5 mL DNA LoBind tubes containing the beads on a 1.5 mL magnetic stand

15 Wait until liquid in tubes is clear, takes about 00:01:00 to complete

16 Gently remove supernatant with 1 mL pipette without disturbing the beads - the LoBind tube remains on the magnetic stand

Note

During washes ensure that all liquid is expelled from the tip as to minimize bead loss

17 Remove first tube from magnetic stand and resuspend beads in at least 600 µL Water buffer, gently pipette up and down at least 5 times to resuspend beads and place the tube On ice

Note

For washes and resuspension the volume of the TE/TW and Water buffers is not important as long as it is at least 300µl, allowing the beads are fully immersed

18 go to step #14 Repeat Step 17 with the other three tubes

19 Place the four 1.5 mL with washed BD Rhapsody beads on 1.5 mL magnetic stand

20 go to step #14 Repeat from Step 14 with TE/TW buffer processing one tube at the time as before

21 Resuspend the beads in at least 600 µL TE/TW buffer and place On ice

Step 3 modification of full vial of RoCKseq beads: T4 polymerase elongation 30m

22 Place the four 1.5 mL tubes with washed BD Rhapsody beads on 1.5 mL magnetic stand and wait until liquid is clear, takes about 00:01:00 to complete

> Note
>
> **IMPORTANT:** During the incubation of the beads on a MacsMix rotator, regularly check that no bubbles form in the Eppendorf tubes. This could lead to the formation of two separate “reaction chambers”, insufficient mixing of the components and eventually incomplete bead modification. In case a bubble forms, remove the tube from the rotator and remove the bubble by inverting the tube until the bubble has shifted and place the tube back onto the rotator.

23 Remove supernatant from first tube - the tube remains on the magnetic stand

24 Resuspend beads from first tube with T4 polymerase mix (from Step 4) by gently pipetting up and down at least 5 times

25 Place the tube on a rack (non magnetic) at Room temperature

> Note
>
> Placing beads in the respective mix back on ice may inhibit the enzymatic reaction.

26 go to step #23 Repeat Steps 23-25 with the remaining three tubes

27 Mix splint (from Steps 10-11) by pipetting with a 200 µL pipette set to 30 µl

28 To each of the four tubes with beads containing the T4 polymerase mix add 6.3 µL of splint, using a new pipette tip each time

29 Place the tubes with resuspended beads into the thermomixer at 37 °C and shake for 00:05:00 at 300 rpm

30 After the incubation at Step 29, place the tubes on a (non-magnetic) rack at Room temperature

31 Add 6.3 µL T4 polymerase to each of the four tubes

> **Note**
>
> Use a fresh (filter) tip each time to avoid contaminating the enzyme stock

32 Place the tubes on a MacsMix tube rotator for 00:10:00 and rotate on second speed setting (at 16 rpm)

> **Note**
>
> The MacsMix tube rotator allows for the beads to be fully mixed during the 10 minutes

33 Transfer the tubes to a thermomixer at 75 °C for 00:10:00 without shaking

> **Note**
>
> This step is critical to inactivate the T4 polymerase

34 During the 10 minutes incubation time in Step 33, **prepare lambda exonuclease mix**: in four 1.5 mL DNA LoBind tubes, pipette 95 µL lambda exonuclease buffer, 832 µL water in

each tube and place On ice . Once the incubation at Step 33 is finished, place the tubes On ice for 00:01:00

35 Wash BD Rhapsody beads as described above in the section **Washing BD Rhapsody Beads** (go to step #14), after which resuspend in at least 200 µL TE/TW buffer and place On ice

Step 4 modification of full vial of RoCKseq beads: lambda exonuclease digest 45m

36 Place the four tubes containing the beads on a 1.5 mL magnetic stand, wait for 00:01:00 and remove the supernatant, not disturbing the beads. The tubes remain on the stand.

37 Remove the first tube from the stand and resuspend the beads using the lambda exonuclease mix (927 µL , from Step 34)

38 Place the tube on a non-magnetic rack at Room temperature

> **Note**
>
> Placing beads in the respective mix back on ice may inhibit the enzymatic reaction

39 go to step #37 Repeat Steps 37-38 with other three tubes

40 To each of the four tubes with beads resuspended in lambda exonuclease mix add 21 µL of lambda exonuclease

> **Note**
>
> Use a fresh (filter) tip each time to avoid contaminating the enzyme stock

41 Transfer the four tubes to a thermomixer at 37 °C for 00:30:00 without shaking

42 Transfer the tubes to a thermomixer set to 75 °C for 00:10:00 without shaking

> **Note**
>
> This step is critical to inactivate the lambda exonuclease

43 After the incubation at Step 42, immediately place the tubes On ice for 00:01:00

44 Wash BD Rhapsody beads as described above in the section **Washing BD Rhapsody Beads** (go to step #14), after which resuspend in at least 200 µL TE/TW buffer and place On ice

Step 5 modification of full vial of RoCKseq beads: final resuspension beads and storage

5m

45 Place the four tubes containing the BD Rhapsody beads on the 1.5 mL magnetic stand and wait for 00:01:00

46 Remove supernatant from first tube and resuspend the beads in 250 µL TE/TW buffer by gently pipetting up and down at least 5 times and place On ice

47 go to step #46 Repeat step 46 with the other three tubes

48 Pool the resuspended beads into a new 1.5 mL Lobind tube

49 Store RoCKseq modified beads at 4 °C . Beads are stable over time in TE/TW buffer.

> **Note**
>
> The fluorescent assay can be performed at a later time point or directly after the bead modification.

Note

STOPPING POINT: BD Rhapsody beads are stable over time similar to unmodified beads when kept in TE/TW buffer and stored at 4 °C

Fluorescent assay for the detection of RoCKseq modification and integrity of DNA oligos on beads

1h

50 Recommended conditions for fluorecent assay

	Condition	Beads	Fluorescent oligo
	Positive control dT	Barcoded beads (unmod)	polyA fluo oligo
	Positive control TSO	Barcoded beads (unmod)	TSO fluo oligo
	Negative control	Barcoded beads (unmod)	Fluo oligo for modification
	RoCKseq beads	Barcoded beads (modified)	Fluo oligo for modification
	dT control RoCKseq beads	Barcoded beads (modified)	polyA fluo oligo
	Unmodified beads	Barcoded beads (unmod)	-----

Note

IMPORTANT: The dT control on RoCKseq beads should be performed as it gives information on the integrity of the dT oligos on the beads, which are needed for polyA capture during the scRNAseq experiment

51 Preheat a thermomixer to 46 °C

52 Prepare TE/TW and Water buffers in 50 mL Falcons and place On ice

Note

If the fluorescent assay is being performed directly after RoCKseq bead modification, the same TE/TW and Water buffers can be used; otherwise make fresh buffers

53 Thaw fluorescent oligos (100 µM) at Room temperature

> **Note**
>
> Keep fluorescent oligos in the dark whenever possible (for example covered in aluminum foil)

54 - **Prepare lysis buffer** in a 1.5 mL Eppendorf tube and place On ice : per sample add 1 µL 1 M DTT (part number 650000063, BD Rhapsody™ Enhanced Cartridge Reagent Kit) to 188 µL µl BD Rhapsody lysis buffer (part number 650000064, BD Rhapsody™ Enhanced Cartridge Reagent Kit) and mix thoroughly

55 Dilute 10 µL of fluorescent oligo (100 µM) 1:10 in ddH_2O and place On ice . Keep in the dark

> **Note**
>
> The diluted fluorescent oligo is stable in the dark at -20 °C

56 Place previously modified BD Rhapsody barcoded beads On ice

57 Wash unmodified beads used as controls as described in **Step 2 modification of full vial of RoCKseq beads: washing BD Rhapsody beads** (go to step #14) and place On ice

58 Pipette 20 µL of RoCKseq modified beads per condition in a new Eppendorf tube and place On ice

59 Place the tubes containing the 20 µL of beads on a 1.5 mL magnetic stand, wait for 00:01:00 and remove the supernatant, not disturbing the beads. The tubes remain on the stand.

60 Add 188 µL lysis buffer + DTT (from Step 54) per condition and place the tube on a non-magnetic rack at room temperature

Note

IMPORTANT: after the addition of the lysis buffer beads should be kept at room temperature. Placing back on ice may increase the fluorescent signal measured at the FACS analyser

61 Add 8 µL of the 10 µM fluorescent oligo per sample (prepared at Step 55) and gently pipette up and down to mix

62 Incubate samples for 00:30:00 at 46 °C shaking at 300 rpm in the dark (for example covering the thermomixer block with aluminum foil)

63 Wash beads as described in **Step 2 modification of full vial of RoCKseq beads: washing BD Rhapsody beads** (go to step #14) and resuspend in 300 µL TE/ TW buffer

64 Strain beads in a Falcon 5 mL Round Bottom Polystyrene Test Tube, place On ice , keep in dark and measure fluorescent intensity at a FACS analyser.

Vortex beads before loading the sample. If the event rate drops, stop acquisition and vortex beads again.

Measure 1000 events per sample.

Note

The final volume into which the beads are resuspended before FACS analysis can vary but at least a volume of 300 µl should be used. Higher volumes will lead to longer analysis times and may require multiple vortexing steps during the acquisition

Note

The beads can be vortexed as they are not used in scRNAseq experiments and are later discarded

Note

The fluorescent signal from the beads should be measured directly after the fluorescent assay

65

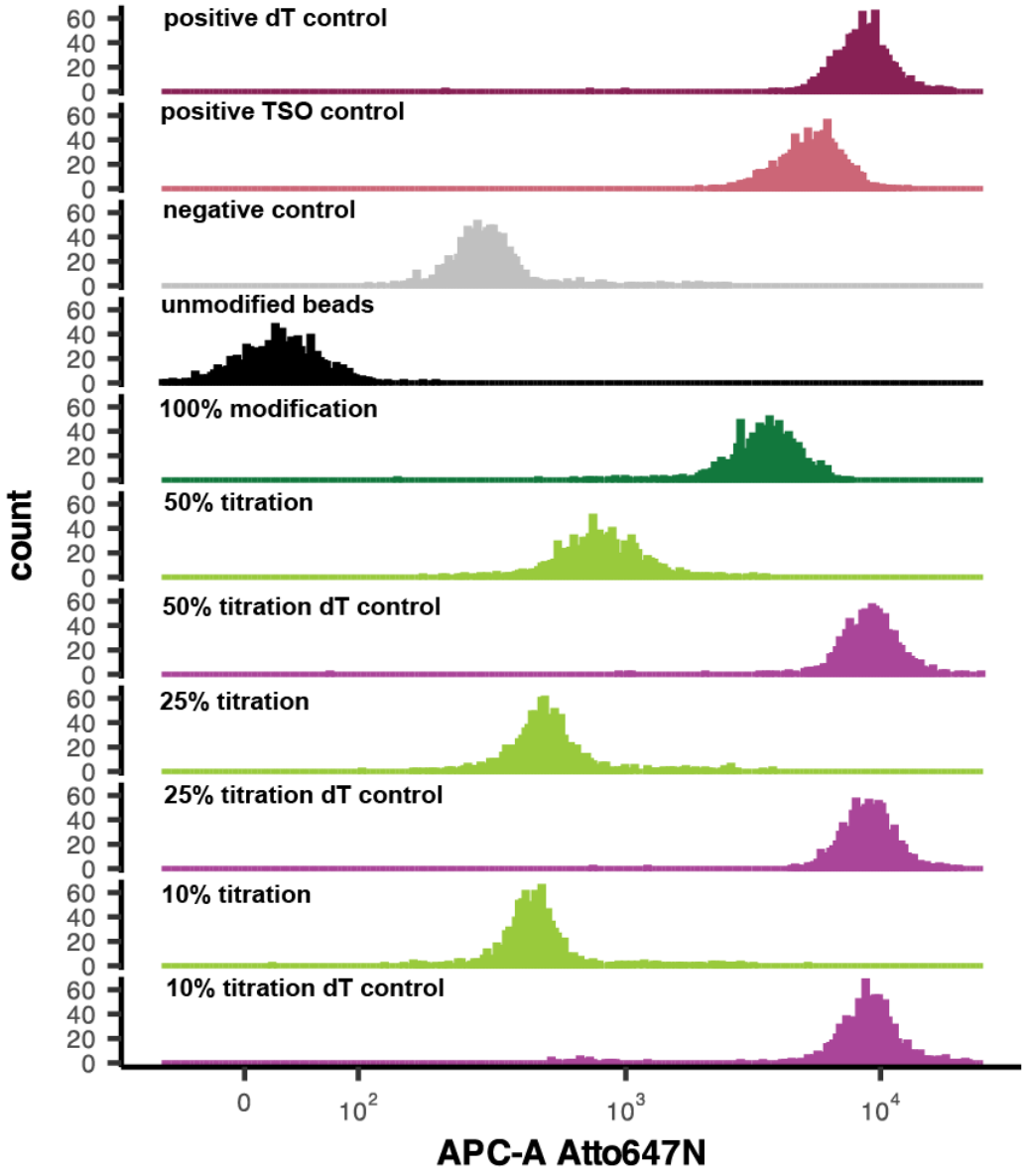
Expected result

The positive controls for the TSO and dT oligos should have a stronger fluorescent signal compared to the negative control and unmodified beads. The negative control may show a certain fluorescent signal as the fluorescent oligo complementary to the modification may bind to cell barcode and UMI sequences.

The FSC-A and SSC-A of the RoCKseq modified beads should be comparable to the one of unmodified beads.

The dT signal from the RoCKseq modified beads should be similar to the dT control.

If titration of modification is performed, the signal will be lower than the one for 100% modification.



Example of titration of RoCKseq bead modification.
Modification was titrated to 50%, 25% and 10% and compared to 100% modification. The dT control on modified beads indicates integrity of the dT olio's on the beads.

Design of ROIseq primers

66 ROIseq primers should be designed **directly 5'** (max. 10bp upstream) to the region of interest (ROI). The length of the primers is 12 nucleotides. Since 12 nucleotides will be included in the cDNA sequencing read (HTS), the ROIseq primer must be in close proximity to the ROI.

Depending on the ROI to be detected, it may be advantageous to position the ROIseq primer further upstream to the sequence of interest. This is the case for example for fusion transcripts, in which having a longer stretch to map on both sides of the fusion breakpoint is beneficial. In this case we recommend using longer read length and placing the ROIseq primer **20-30 bp upstream** to the ROI itself.

The ROIseq primer has the following structure:

5'-TCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNN-3', the N being the sequence of the ROIseq primer which identical to the coding strand.

An additional consideration when designing ROIseq primers is that ideally the read generated after ROIseq priming should be unique, i.e. doesn't map to multiple loci.

ROIseq primers should be ordered in HPLC grade and at 0.2 µmol scale and resuspended in DNA Supension buffer from Teknova (T0221).

RoCK and ROI library generation

67 RoCK and ROI library generation follows the standard BD Rhapsody workflow (mRNA capture, reverse transcription and exonuclease treatment: Doc ID: 210966; library generation Doc ID: 23-21711-00) with the following adaptations (steps 67.1-67.4 indicate the steps in the standard protocols where the changes occur):

67.1 **Resuspending barcoded beads prior to loading on cartridge:** to account for the bead loss during modification, resuspend the RoCKseq beads in 680 µL Sample Buffer (Cat. No. 650000062, BD Rhapsody™ Enhanced Cartridge Reagent Kit) instead of 750 µL prior to loading on the BD Rhapsody cartridge

67.2 **Random priming and extension**: if a single ROIseq primer is added, dilute 1 µL of the 100 µM primer 1:10 in ddH_2O and pipette 4 µL of the diluted mix during the **Random Priming and Extension step** (after pipetting the 174 µL). Add the ROIseq primers after the beads are resuspended in the **Random Primer mix**.

If multiple ROIseq primers are used, mix 1 µL of each ROIseq primer (100 µM), add ddH_2O up to 10 µL and add 4 µL to the mix.

67.3 **RPE PCR:** add 1 µL of 100 µM T primer to each sample after the **RPE PCR mix** is added to the **Purified RPE product**.

Note

Adding the primer after mixing of the RPE PCR mix and Purified RPE product ensures that each sample receives the same amount of T primer when working with multiple samples

67.4 **Indexing PCR**: for indexing of RoCKseq libraries, a separate PCR is performed substituting 5 µL of the Library Forward Primer (BD Rhapsody™ Enhanced Cartridge Reagent Kit, part number 91-1085) with 5 µL of 100 µM of a custom indexing primer. The same primary library and reverse primers are used as recommended by the manufacturer. The reaction is thus as follows:

For WTA library (from BD Rhapsody Doc ID: 23-21711-00):

	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 2 libraries with 10% overage (µL)
	PCR MasterMix (Cat. No. 91-1118)	25	30	55
	Library Forward Primer (Cat. No. 91-1085)	5	6	11
	Library Reverse Primer (1-4) (Cat. Nos. 650000080, 650000091-93)	5	6	–
	Nuclease-free water (Cat. No. 650000076)	5	6	11
	Total	40	48	77

For TSO library:

	Reagent	For 1 library (µL)	For 1 library with 20% overage (µL)	For 2 libraries with 10% overage (µL)
	PCR MasterMix	25	30	55

	Reagent	For 1 library (µL)	For 1 library with 20% overage (µL)	For 2 libraries with 10% overage (µL)
	(Cat. No. 91-1118)			
	T primer + adapter	5	6	11
	Library Reverse Primer (1-4) (Cat. Nos. 650000080, 650000091-93)	5	6	–
	Nuclease-free water (Cat. No. 650000076)	5	6	11
	Total	40	48	77

IMPORTANT: RoCKseq and dT-based libraries of a given sample should be indexed with the **SAME** BD Rhapsody Library Reverse Primer and will thus have the same 8 bp index. The two data modalities are then separated bioinformatically (see Step 69)

Note

Until this step the dT and TSO libraries are in a single reaction, while at this step they are separated.

67.5 **If no ROIseq is being performed omit step 67.2**

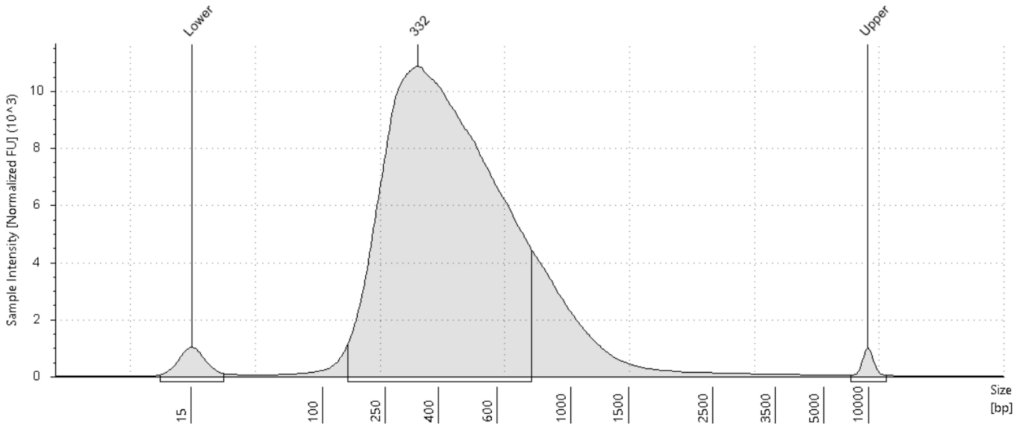
68

Expected result

We recommend checking the library sizes of primary and indexed libraries. The library sizes and concentrations for RoCKseq and RoCK and ROI libraries should not differ from standard BD Rhapsody libraries.

Expected result

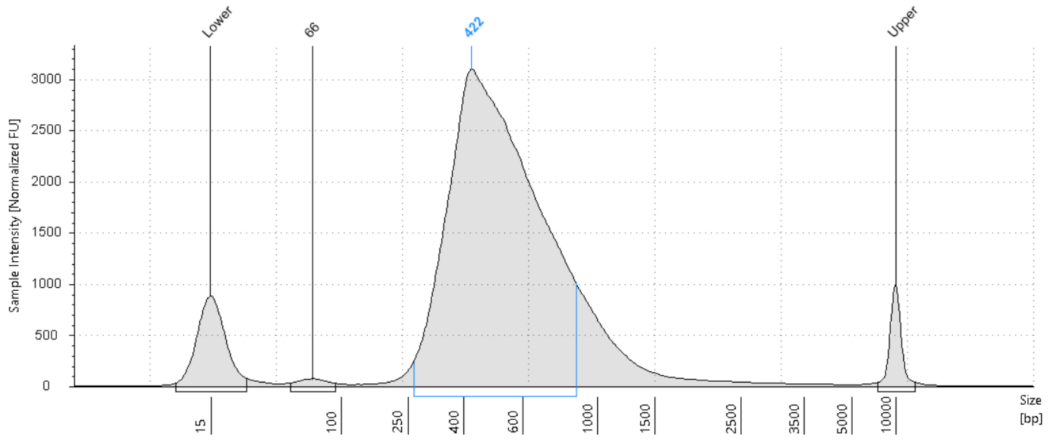
Example of primary library size



Tape station trace measured with High Sensitivity D5000 tape for RoCK and ROI

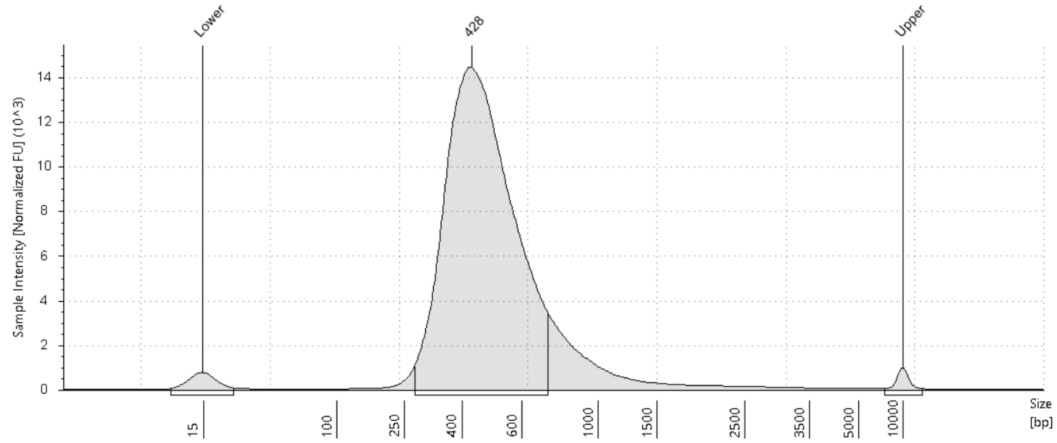
Expected result

Example of indexed library size (WTA library derived from dT oligos)



Tape station trace measured with Agilent High Sensitivity D5000 tape for RoCK and ROI

Example of indexed library size (TSO library)



Tape station trace measured with Agilent High Sensitivity D5000 tape for RoCK and ROI

Sequencing

68.1 We recommend pooling the WTA and TSO libraries in a 1:1 ratio.

For sequencing of pooled libraries including at least one RoCKseq modified sample (with or without ROIseq primers), a **custom R1 primer** should be spiked in (see Materials).

The length of R1 should be 60 bp, while the length of R2 may vary depending on the ROI of interest (see section **Design of ROIseq primers,** Step 66). We recommend using an R2 of 62 bp for ROIs such as point mutations and splice junctions and an R2 of 150 bp for fusion breakpoints and CRISPR target sites.

Data analysis

69 RoCK and ROI data can be analysed using our custom pipeline, found at https://zenodo.org/records/11070201 under the GPLv3 terms. For downstream data processing please see https://zenodo.org/records/11124929.