SIGNAL-seq: Library Preparation Protocol (v1.0)

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Projected Experimental time: 1 full day (Library preparation)
Start the Library prep day by 09:00

Notes:

- Several buffer components require thawing, i.e. 5x Maxima RT buffer take these out in advance and leave on ice to prevent delays.

Reagents to Generate on Previous Day:

- 100 mM PMSF stock solution in isopropanol.

2x B+W (Store in -20)

Reagent	Stock Concentration	Final Concentration	Volume (µL)
1 M Tris-HCL pH 8.0	1 M	10 mM	50
5 M NaCl	5 M	2 M	2000
EDTA 0.5 M	0.5 M	1 mM	10
H_2O	NA	NA	2940
Total Volume			**5000**

1x B+W-T (Store in -20)

Reagent	Stock Concentration	Final Concentration	Volume (µL)
1 M Tris-HCL pH 8.0	1 M	5 mM	25
5M NaCl	5 M	1 M	1000
Tween-20 (10 %)	10 %	0.05 %	25
EDTA 0.5 M	0.5 M	0.5 mM	5
H_2O	NA	NA	3945
Total Volume			**5000**

Reagents to Generate on Day:

N.B. These +RI additions are for 2 sublibraries, for more sublibraries increase the volumes required.

2x B+W + RI – for 2 Sublibraries

Reagent	Volume (µL)
2xB+W	110
SUPERase inhib.	2
Total Volume	**112**

1x B+W-T + RI – for 2 samples

Reagent	Volume (µL)
1xB+W-T	3600
SUPERase inhib.	5
Total Volume	**3605**

Tris-T + RI

Reagent	Volume (µL)
10mM Tris-HCL pH8.0	600
Tween-20 (10%)	6
SUPERase inhib.	1.5
Total Volume	**607.5**

10 mM PMSF

Reagent	Volume (µL)
100 mM PMSF	1
Isopropanol	9
Total Volume	**10**

2.1 - Binding cDNA Lysates on Beads

Wash the MyOne C1 Dynabeads

Notes:

- Remove MyONE C1 Dynabeads from the fridge and equilibrate to room temperature for 30 minutes before using.
- Remove PMSF from the -20C freezer, thaw and vortex to re-dissolve any remaining precipitate.
- The current setup requires a 1.5 mL DynaMag rack to process the ADT library at the SPRI cleanup stage. Volumes can be reduced to fit on a smaller PCR tube Magnetic rack.

1. For each lysate to be processed, add 44 µL of MyOne C1 Dynabeads to a 1.5 mL protein lo-bind tube (eg, 1 lysate=44µL, 2 lysates = 88µL, 3 lysates = 132µl etc. .)
2. Add 400µL of 1xB&W-T buffer, resuspend
3. Place the sample against a magnetic rack and wait until liquid becomes clear (1-2 min).
4. Remove supernatant and resuspend beads in 400µL of 1xB&W-T buffer (at least 100µL per sublibrary).
5. Repeat steps 3-4 two more times for a total of 3 washes.
6. Place the sample against a magnetic rack and wait until liquid becomes clear.
7. Resuspend beads in 55µL (per sample) 2xB+W buffer + RI.

Streptavidin Bead binding

1. Remove the sublibraries from the -80C freezer and incubate at 37C on the thermocycler for 5 minutes (lid temperature 50C), check that the solution is clear and that all precipitate has dissolved. If some precipitate remains, vortex the tubes briefly and heat for another minute.
2. Add 2.5 µL of **10 mM** PMSF to each sample, mix 5 times with a P200 pipette and briefly Centrifuge (~1 sec) the lysates and leave at room temperature for 10 mins.

3. Add 55 µL of the binder beads in 2xB+W to each sublibrary lysate. Mix 5x with a pipette set to 90 µL. Do not lose any dead volume in the pipette tip as this will negatively affect the sublibrary data.
4. Shake the sublibrary tubes at RT for 60 minutes. Place the tubes in a 96 well plastic plate holder taped to a vortex. We set the Vortex Genie to setting 3.
5. Thaw 5X RT buffer and make sure you have some Ultrapure water ready at RT.
6. Remove the tubes from the shaker, vortex the tube briefly and then ~1 sec centrifuge without letting the step-beads settle at the base of the tube.
7. Place the tubes against a PCR tube magnetic rack (high-setting) and wait for all the beads to bind the magnet and the **solution to become clear** (should take ~1-2mins).
8. Remove the clear supernatant with a pipette and discard, while keeping the tubes in the magnetic rack.
9. Remove the tubes from the magnetic rack and resuspend beads with 125 µL of 1xB+W-T +RI. Take care not to lose any dead volume in the pipette tip.
10. Incubate tubes at RT for 1 minute.
11. Place the tubes back against the magnetic rack (high-setting) and wait for the solution to turn clear.
12. Remove the clear supernatant with a pipette and discard, while keeping the tubes on the magnetic rack.
13. Repeat **steps 9-11** for a second wash with 1xB+W-T + RI.
14. Remove the tubes from the magnetic rack and resuspend beads in 125 µL of Tris-T + RI.
15. Incubate the beads for 1 min at RT and place on ice. Proceed directly to: **2.2 Template Switch Reaction.**

2.2 Template Switch Reaction

1. Make the Template switch Master mix, scale the Master mix to the number of sublibraries to be processed:

Reagent	1x (µL)	2x (µL)
Water	44	88
Maxima RT Buffer (5x)	22	44
Ficoll PM-400 (20%)	22	44
10mM dNTPs (each, total 40mM)	11	22
Merck RNase inhibitor	2.75	5.5
TSO (BC_0127)	2.75	5.5
Maxima RT H minus Enzyme	5.5	11
Total	**110**	**220**

2. Place the sample against the magnetic rack and wait until the tube becomes clear.
3. With the tube still on the rack, remove the supernatant and wash with 125 µL of Ultrapure Water (at RT) for 1 minute. Do not resuspend beads.
4. Remove supernatant and discard.
5. Resuspend beads off the magnetic rack with 100 µL Template Switch Mix, mix thoroughly with a P200 pipette, the solution is very viscous from the Ficoll.
6. Incubate at RT for 30 mins with agitation on the vortex.

7. Incubate in thermocycler with the following program:

Run time	Lid Temp	Sublib Volume
90min	70C	100 µL
Step	**Time**	**Temperature**
1	90 min	42C
2	Hold	4C

2.3 cDNA Amplification

1. While the Template reaction proceeds make the cDNA PCR amplification reaction buffer:

Reagent	1x (µL)	2x (µL)
Kapa Hifi 2x MM	60.5	121
BC_0108 (10 µM)	4.84	9.68
BC_0062 (10 µM)	4.84	9.68
Water	50.82	101.64
Total	**121**	**242**

2. **If you are performing SIGNAL-seq protein detection, you need to spike in the ADT_cDNA primer. Otherwise proceed directly to step 3.**
   a) **Prepare the ADT_cDNA primer to 2 µM (1:50 in water if 100 µM stock)**
   b) **Add 0.99 µL of 2 µM primer stock per sub library, subtracting an equal volume of water from the PCR master mix.**

3. Place the sample against a magnetic rack. Wait for the solution to clear of beads and remove the supernatant (~1-2mins).
4. With the bead sample remaining on the rack, wash the beads with 125 µL of water, leave for 1 minute.
5. Remove supernatant and discard.
6. Remove from the rack and resuspend the beads completely in 110 µL of cDNA amplification master mix.

7. **Run the cDNA amplification process:**

Note, these cycle numbers are determined on several cell lines (HeLa-human and MCF-1 mouse) and on several primary organoid cell types (CRC PDO lines-human, LGR5^{+} SI organoids-mouse) for primary cells. These cycle numbers can be different depending on the cell type

and sample type on input. We recommend running an amplification cycle number pilot with several spare sublibraries to see the amplification levels by Qbit and bioanalyzer.

Cell Number	First Stage	Second stage (Cell lines)	Second stage (Primary cells)
250 - 1000	5	13	15
1000 – 2000	5	11	12
2000 – 6000	5	9	10
6000 - 12500	5	7	8

8. Incubate in the thermocycler with the following program:

Run time	**Lid Temp**	**Sublib Volume**
40-70 min	105C	110 µL
Step	**Time**	**Temperature**
1	3 min	95C
First Stage		
2	20 sec	98C
3	***45 sec**	**65C**
4	3 min	72C
Go to step 2, repeat 4 time (5x cycles total)		
Second Stage		
5	20 sec	98C
6	***20 sec**	67C
7	3 min	72C
Go to step 5, repeat for X total cycles		
8	5 min	72C
9	Hold	4C

NOTE: If continuing on with the protocol remove SPRI beads from 4C and warm to RT.

Possible stopping point – Remove tubes from the thermocycler and store at 4C overnight in the fridge. Continue to the next step on AM of the following day.

If you are performing SIGNAL-seq with ADT staining proceed to section 2.3 - ADT Library SPRI Preparation immediately (below) otherwise proceed to section 2.4 RNA Library SPRI preparation.

2.3 - ADT Library SPRI cleanup

After cDNA amplification the ADT and mRNA libraries can be size separated by SPRI beads after this section, continue with **2.4 RNA Library SPRI cleanup**.

1. Prepare 85% EtOH, remove SPRI beads from the fridge and bring to room temp.
2. Place tubes against the magnetic rack and wait for the solution to clear as the beads bind the magnet.
3. Transfer 100 µL of supernatant into a new PCR-clean tube.
4. Add 60 µL of SPRI beads (0.6x) to the cDNA amplification mix and incubate for 5 minutes at RT to bind cDNA molecules. **N.B: DO NOT DISCARD THE SUPERNATANT FROM THIS REACTION AS THIS CONTAINS THE ADT LIBRARY.**
5. The supernatant contains the ADT library, and the beads will have bound the RNA library. Place the tube on the magnet and wait ~2 minutes for the solution to turn completely clear.
6. Carefully take all 160 µL of supernatant and transfer to a new labelled **1.5 mL** PCR-clean tube. Use a P10 pipette to remove any extra remaining supernatant that might lead to impurity in the RNA library.
7. Wash the RNA library on the SPRI beads with 180 µL 85% EtOH, do not resuspend beads. Wait for 1 minute.
8. Using a P200 and then a P10 pipette, aspirate and discard the ethanol from each tube.
9. Repeat **step 7-8** for a second wash.

10. Centrifuge tube briefly (~1 sec) to bring remaining ethanol that might be stuck on the tube wall to the bottom of the tube.
11. Return to magnet, remove ethanol and air-dry beads (~2-3 mins). Be careful not to let the beads over dry and crack.
12. Resuspend beads from each tube in 50 µL of water. Once beads are fully resuspended in the water, incubate the tube at 37C for 10 mins to elute the RNA cDNA library.
13. Bind tubes against the magnetic rack and wait until liquid becomes clear (~1-2mins).
14. Transfer 50 µL of eluant into a new labelled optical grade PCR tube. This is the **RNA library**, place this tube at 4C and save until **Section 2.4, mRNA Library preparation**.
15. Add 1.4x SPRI to the ADT library (Supernatant transferred in **step 4** to obtain a final SPRI volume of 2x SPRI. Here the cDNA PCR reaction volume is 100 µL, 60 µL (0.6x SPRI) was added to separate the ADT/RNA libraries in **step 4**. Therefore, adding another 140 µL of SPRI to the transferred supernatant. The final volume will be 300 µL (100 µL supernatant : 200 µL SPRI).
16. Incubate 10 minutes at room temperature.
17. Place the tube on the magnetic rack and wait for ~1-2 minutes, wait for the solution to become completely clear.
18. With first a P200 and then a P10 pipette carefully remove and discard the supernatant.
19. Add 400 µL of 80% EtOH to the tube without disturbing the pellet and let stand for 1 min for a single ethanol wash.
20. Carefully discard the ethanol with a P200 and then a P10 pipette.
21. Centrifuge tube briefly (~1 sec) to bring remaining ethanol that might be stuck on the tube wall to the bottom of the tube.
22. Place back on the magnetic rack and remove remaining ethanol with a P10 pipette.
23. Resuspend the 50 µL in water and transfer entire contents to a 200 µL PCR-clean PCR tube.

24. Perform a second round of 2x SPRI cDNA purification by adding 100 µL SPRI reagent directly to the tube and resuspend the beads, mix thoroughly.
25. Incubate for 10 minutes at room temperature.
26. Place the tube on the magnetic rack and wait ~1-2 mins for the solution to completely clear.
27. With first a P200 and then a P10 pipette carefully remove and discard the supernatant.
28. Add 185 µL of 85% EtOH to the tube whilst still on the magnet without disturbing the pellet and stand for 1 minute.
29. With first a P200 and then a P10 pipette carefully remove and discard the supernatant.
30. Add 185 µL of 85% EtOH to the tube whilst still on the magnet without disturbing the pellet and stand for 1 minute.
31. Centrifuge tube briefly (~1 sec) to bring remaining ethanol that might be stuck on the tube wall to the bottom of the tube.
32. Place back on the magnetic rack and remove remaining ethanol with a P10 pipette, allow the beads to air dry for 2 mins, (do not allow the beads to over dry and crack).
33. Resuspend the beads in 90 µL of water, pipette vigorously to ensure complete resuspension.
34. Incubate at RT for 5 minutes.
35. Place the tube on a magnetic rack and wait ~1-2 mins for the solution to become completely clear. Transfer clear supernatant into two new labelled PCR tubes, splitting the sample in half (45 µL in each tube).
36. Store one tube in at -20C as a backup tube or for further PCR cycle optimization. Take one tube forwards for ADT sequencing library generation.

37. Prepare the following 100 µL library PCR reaction:
    a) Here the SPLiT-seq barcode primer contains the 4th sublibrary i7 barcode and so each sub library requires a unique barcode if they are to be sequenced as a pool.

b) **IT IS CRITICAL THAT THE i7 INDEX IS RECORDED AT THIS POINT!** If you intend to pool the ADT libraries with the RNA libraries that will be generated in the next step then each individual library requires a unique index.

Reagent	**1x (µL)**
Kapa Hifi 2x MM	50
Purified ADT cDNA (from step 34)	45
ADT_lib (10 µM)	2.5
One of BC_0076 – BC_0083 (10 µM)	2.5
Total	**100**

38. Place tube in thermocycler and run the following program alter the cycle number depending on your experimental conditions:

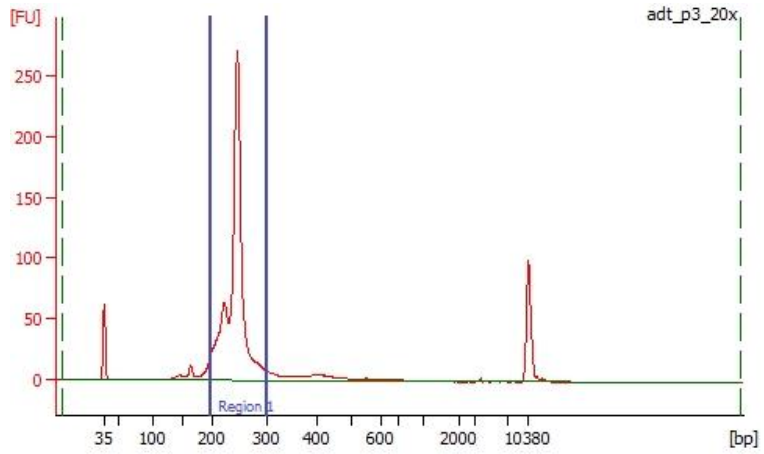
Run time	**Lid Temp**	**Sublib Volume**
30-45 min	105C	100 µL
Step	**Time**	**Temperature**
1	3 min	95C
2	20 sec	95C
3	30 sec	60C
4	20 sec	72C
Go to Step 2, repeat 5-11 times (6-12x cycles total)		
5	5 min	72C
6	Hold	4C

39. Purify the PCR product using a 1.2x SPRI purification by adding 120 µL of SPRI reagent, mixing thoroughly.
40. Incubate for 10 minutes at room temperature.
41. Place the tube on the magnetic rack and wait ~1-2 mins until the solution is completely clear.
42. With first a P200 and then a P10 pipette carefully remove and discard the supernatant.
43. Add 250 µL of 85% EtOH to the tube without disturbing the pellet and let the tube stand for 1 min.

44. With first a P200 and then a P10 pipette carefully remove and discard the supernatant.
45. Add 250 µL of 85% EtOH to the tube whilst still on the magnet without disturbing the pellet and stand for 1 minute.
46. Remove the clear supernatant and centrifuge tube briefly (~1 sec) to bring remaining ethanol that might be stuck on the tube wall to the bottom of the tube.
47. Place back on the magnetic rack and remove remaining ethanol with a P10 pipette, allow the beads to air dry for 2-3 mins, (do not allow the beads to over dry and crack).
48. Resuspend the beads in 25 µL of water, pipette vigorously to ensure complete resuspension.
49. Incubate at RT for 5 minutes.
50. Place the tube on the magnetic rack and wait ~1-2 mins for the solution to become completely clear. Transfer clear super into a new labelled PCR tube, this is the Illumina compatible ADT library.
51. ADT libraries are now ready for QC and sequencing. Quantify libraries by QuBit and size profile with BioAnlayzer.

    NOTE: Quantifying ADT library concentration can be challenging by bioanalyzer. It can be beneficial to quantify the libraries by qPCR with p5/p7 primer sets to get a more accurate concentration.

Sample Bioanalyzer trace:



The ADT libraries will have the following structure:

Read 1 - 15nt (covering Ab barcode)
i7 - 6nt (PCR i7 BC4)
Read 2 - 86nt (Cell Barcodes RT1, L2, L3 and UMI)

Read 2 will have the following structure UMI: 1-10 bp, BC3 (L3): 11-18 bp, BC2 (L2): 49-56 bp, BC1 (RT1): 79-86 bp.

Proceed to Section 2.4 – RNA Library SPRI Preparation.

2.4 – RNA Library SPRI Preparation

Skip section 2.3 and proceed to 2.4 for single RNA modality only SPLiT-seq RNA library cDNA purification.

Notes:

- Remove SPRI beads from 4C and equilibrate to RT for 30 minutes before using.

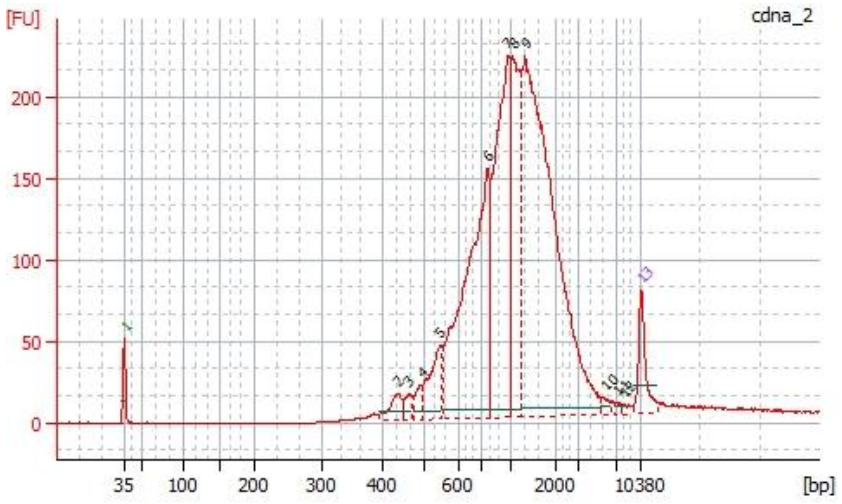
1. Prepare 85% ethanol
2. If you are running an ADT multimodal library continue with **step 3**. However, if you are running a RNA only library, continue with steps 2a. – o. to before going to step 3.

    a. Place the tubes with amplified cDNA against the magnetic rack and wait for the beads to bind the magnet (~2 mins).
    b. Transfer 100 µL of supernatant into a new PCR-clean tube.
    c. Add 80 µL of SPRI beads (0.8x) to the PCR tube containing supernatant, vortex briefly and spin down for ~1 sec.
    d. Incubate at room temperature for 5 minutes.
    e. Place the tubes against the magnetic rack and wait for the beads to bind the magnet for ~2 minutes.
    f. With first a P200 and then a P10 pipette carefully remove and discard the supernatant.
    g. Without resuspending beads add 180 µL of 85% EtOH and incubate at RT for 1 minute.
    h. With first a P200 and then a P10 pipette carefully remove and discard the supernatant.
    i. Add 180 µL of 85% EtOH to the tube whilst still on the magnet without disturbing the pellet and stand for 1 minute. Repeat this process for a total of 2x EtOH washes.
    j. Remove clear EtOH supernatant.
    k. Centrifuge tube briefly (~1 sec) to bring remaining ethanol that might be stuck on the tube wall to the bottom of the tube.
    l. Place back on the magnet (low-setting) and remove remaining ethanol with a P10 pipette, allow the beads to air dry for 2-3 mins with the lid open.

m. Resuspend the beads in 50 µL of water, pipette vigorously to ensure complete resuspension.
n. Incubate at 37C for 10 minutes.
o. Place the tube on the magnet (low-setting) and wait ~1 min for the solution to become completely clear. Transfer clear supernatant into a new labelled PCR tube.

3. Repeat SPRI 0.8x clean-up procedure 2a.-o. for a second clean-up to remove any residual primer, **elute the sample in 20uL of water at the end**.
4. Run 1 µL of the sample on the Qubit dsDNA HS to determine concentration.
5. Run 1 µL of the amplified cDNA on the bioanalyzer DNA HS platform to check for primer dimer, you will probably have to dilute the sample 1:5-1:10 seems to work, there should ideally be none, but >95% purity (no primer or primer dimer) should be acceptable - see traces below for examples.
6. Sublibraries can now be stored at -20C for a week or -80 for a month, if proceeding within 48hrs to section **2.5 RNA Illumina Nextera Tagmentation,** store the sublibraries at 4C.

Sample Bioanalzyer trace:



2.5 RNA Illumina Nextera Tagmentation

1. Thaw TD tagment buffer at RT and vortex

2. Dilute cDNA libraries to 0.2 ng/µL in water, **use the Qubit concentration readings**.

3. Run the following protocol on the thermocycler once the tagment reaction mix is created in **Step 4**. Since the tagment enzyme is active at RT it is important to have this protocol prepared beforehand to activate immediately. The program is as follows:

Run time	**Lid Temp**	**Sublib Volume**
6 min	100C	20 µL
Step	**Time**	**Temperature**
1	5 min	55C
2	Hold	10C

4. Generate the following mix in a PCR tube make sure to do it **in the following order and mix 10x with a P20 set to 15uL**:

Reagent	**1x (µL)**
TD Tagment Buffer	10
cDNA diluted to 0.2 ng/µL in water	5
Tagment DNA Enzyme (tagmentation mix)	5
Total	**20**

5. Centrifuge the mix on a tabletop centrifuge for 15 secs and place immediately on the thermocycler running the tagmentation protocol shown above in **step 3**.
6. When the program reaches 10C, **immediately** proceed to the next step **(7)** as the tagment enzyme is still active.
7. Add 5 µL of NT quenching buffer to each tube, pipette 5x times to mix with a P20 set to 15 µL.

8. Centrifuge for 15 secs on tabletop centrifuge.
9. Incubate at RT for 5 minutes to quench the transposome.
10. Generate the following PCR mix by adding directly to the quenched transposome reaction mix tube:

N.B: It's critical to record the sublibrary index (BC4) that is given to each sublibrary and each sublibrary must have its own index. If you have run the ADT library generation before this, consider which indexes have been assigned to these paired sublibraries.

Reagent	**1x (µL)**
Quenched Transposome mix	25
Nextera PCR mix NPM	15
Water	8
N7 index oligo BC0076-0083 (10 µM)	1
Nextera Oligo BC_0118 (10 µM)	1
Total	**50**

11. Run the following thermocycling protocol:

Run time	**Lid Temp**	**Sublib Volume**
60 min	100C	50 µL
Step	**Time**	**Temperature**
1	3 min	72C
2	30 sec	95C
3	10 sec	95C
4	30 sec	55C
5	30 sec	72C
Go to Step 3, repeat 11 times (12x cycles total)		
6	5 min	72C
7	Hold	4C

12. Transfer as much of the reaction as possible into a PCR strip tube, **note down the exact volume of the transferred PCR mix**. Purify PCR products using SPRI beads with a 0.6x-0.8x double sided selection procedure to generate final libraries.

13. Measure the concentration on the Qubit and the Bioanalyzer for amplicon size. There should be no detectable primer dimer in the library before sequencing. If there is, repeat the 0.8x SPRI selection procedure.

2.5 – Sequencing

1. Prepare a dilutions and final library pool based on the recommendation of the sequencing platform.

2. The libraries must be sequenced with the following configuration, using 5% phiX for RNA or pooled RNA/ADT libraries. We use 7.5% phiX for ADT-only libraries to improve quality.

RNA sequencing format

Read	Cycles
Read 1 (cDNA)	74
I7 index	6
Read 2 (Barcode)	86
i5 Index	0