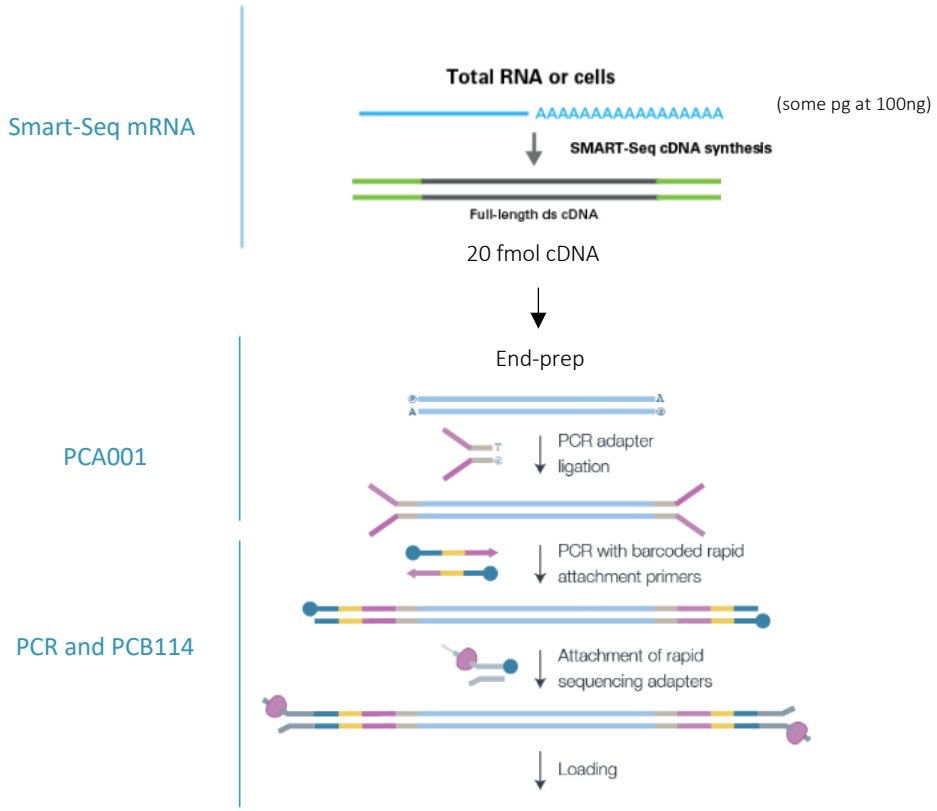
CDNA-NON-ORIENTED-RAP-CHEMISTRY14

LONG-READ CDNA PROTOCOL WITH CDNA AMPLIFICATION BY THE SMAR SEQ MRNA PROTOCOL, PCA ADAPTER LIGATION (EXP-RAP001-ONT) AND PCR MULTIPLEXING WITH THE PCB114-ONT KIT

This document describes the steps to be taken to achieve the platform-optimized non-oriented cDNA library protocol



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Material

- Total RNA: a few pg at 100 ng

Kits

- Smart-Seq mRNA de Takara (634772 ou 634773)
- PCR Adapter (PCA) in the EXP-PCA001 ONT Kit
- ONT SQK-PCB114

Reagents

- NEBNext Ultra II End repair/dA-tailing Module (NEB, cat # E7546)
- NEB Blunt/TA Ligase Master Mix (NEB, cat #M0367)
- LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- Eau Nuclease-free
- Absolute Ethanol

I. Total RNA amplification

Material

- Total RNA: some pg at 100 ng **optimized for 10ng**
- Kit Smart-Seq mRNA de Takara (634772 ou 634773)

Table 1. SMART-Seq mRNA components.

SMART-Seq mRNA	634772 (24 rxns)	634773 (96 rxns)	634770 (384 rxns)
Box 1 (Store at –70°C)			
Control Total RNA (1 µg/µl)	5 µl	5 µl	4 x 5 µl
Box 2 (Store at –20°C)			
SMART-Seq v4 Oligonucleotide (48 µM)	24 µl	96 µl	4 x 96 µl
PCR Primer II A (12 µM)	24 µl	96 µl	4 x 96 µl
5X Ultra® Low First Strand Buffer	96 µl	384 µl	4 x 384 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	48 µl	192 µl	4 x 192 µl
3′ SMART-Seq CDS Primer II A (12 µM)	48 µl	192 µl	4 x 192 µl
RNase Inhibitor (40 U/µl)	60 µl	240 µl	4 x 240 µl
Nuclease-Free Water	2 x 1 ml	4 ml	4 x 4 ml
10X Lysis Buffer*	460 µl	1.85 ml	4 x 1.85 ml
Elution Buffer (10 mM Tris-Cl, pH 8.5)†	2 x 1.7 ml	2 x 6.8 ml	8 x 6.8 ml
SeqAmp™ DNA Polymerase	50 µl	200 µl	4 x 200 µl
SeqAmp CB PCR Buffer (2X)	1.25 ml	5 ml	4 x 5 ml

*Store 10X Lysis Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
†Store Elution Buffer at –20°C. Once thawed, the buffer can be stored at room temperature.

The protocol was optimized for **10ng of total RNA** with **12 cycles of amplification.**
It is possible to use other quantities (from a few pg to 100 ng), but the number of PCR cycles must be adjusted.
The purification step must be carried out with Agencourt AMPure XP beads

Thaw at room temperature:
- 10 X lysis buffer (avoid blowing bubbles when mixing)
- Nuclease free water
- 5X Ultra Low First-Strand Buffer

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Thaw in ice:
- SMART-Seq v4 Oligonucleotide (48 µM)
- RNase Inhibitor (40 U/µl)
- 3' SMART-Seq CDS Primer II A

Take out the SeqAmp DNA Polymerase enzyme, in ice, just before using it

Start the following **Incubation** program : ***3min at 72°C***

Summary of the 1st strand:

Prepare the 10X Reaction Buffer

Volume Responsive	
10X lysis Buffer	19 µl
RNAse Inhibitor	1 µl
Total 20 µl	

Prepare Plate Samples

Volume Responsive	
10 ng total RNA x µl	
Eau nuclease-free	qsp 9,5 µl
10X reaction buffer	1 µl
Total 10,5 µl	

Put the samples on ice

Add to ice, **2 µl** *3' SMART-Seq CDS Primer II A*.
(If the expected number of PCR cycles during cDNA amplification is greater than 17 cycles; add only **1 µL** of 3' SMART-Seq CDS Primer II A).

Mix by patting and centrifuging (final volume of 12.5 µl)

Incubate at 72°C for 3 min then immediately on ice for 2 min (max 10min)

Launch the pre-incubation program at 42°C of the **following FIRSTSD** program :

90min at 42°C
10 min at 70°C,
4°C for ever

During the 3min incubation prepare the **masterMix +10%**

Reagent	**Volume**
5X Ultra LowFirst-strand buffer	4 µL
SMART-seq v4 oligonucleotide (48uM)	1 µl
RNase inhibitor	0.5 µL
SMARTScribe Reverse Transcriptase	2ul

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(add the enzyme just before using the masterMix)
Total **7.5 µl**

Add **7.5 µL of masterMix per well to samples**

Mix by patting and then centrifuge

Place the plate in the thermal cycler and start the FIRSTSD program

The tubes can stay at 4°C overnight

PCR amplification of double-stranded cDNA (after synthesis of the 1st strand):

Thaw all reagents for PCR in ice, vortex, and centrifuge
Take the enzyme out just before use in ice (do not vortex it)

Prepare the PCR Mix for all reactions, + 10%, add in the following order:

Reagent	Volume
2X SeqAmp CB PCR Buffer	25 µL
PCR Primer IIA	1 µL
Nuclease-Free Water	3µl
SeqAmp DNA Polymerase	1 µl
(to be taken out only when added)	
Total 30 µl	

Add **30µl of the PCR mix to the 20µl of the 1st strand of cDNA** (final volume of 50µl)

Mix by gently vortexing and make a short spin

Closing wells with plugs

Centrifuger

Put the program in the thermal cycler previously launched to be at 95°C

AMPLI program (duration approx. 1 hour):

volume final 50 µl

Step	Temperature	Time	Cycle
Initial denaturation	95°C	1 min	1
Denaturation	98°C	10 sec	12
Annealing	**65°C**	30 sec	12
Extension	**68°C**	**3 min**	**12**
Final extension	72°C	10 min	1
Hold 4°C ∞			

The plate can be stored at 4°C overnight

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Purification of cDNA amplified by AMPure XP beads

Bring the AMPure XP beads to room temperature for at least 15 min and vortex them before use.

Remove the Elution Buffer from the freezer and keep it at room temperature.

Prepare 80% fresh EtOH.

Add **50µL of AMPure XP Magnetic Beads**. Vortex well before use.

Mix by pipetting 10x using a multichannel pipette (final volume of 100 µl)

Incubate at **room temperature for 8 min** (to bind the DNA to the beads).

Place the samples on the magnetic carrier **for 5 min** until the solution is completely clear.

Remove the supernatant, leaving the tubes still on the magnetic support.

Add **200µl of fresh 80% EtOH, wait 30sec** then remove the supernatant.

Repeat the wash a second time.

Make a quick spin and after putting it back on the magnet, remove the residual EtOH with a pipette

Leave the tubes open at room temperature for **2/3 min** (**until the beads are dry without cracking).**

Add **17µl of Elution Buffer** (EB=Tris Buffer, HCL10mM ph8.5) at room temperature to the beads.

Mix while pipeting, **incubate for 2 min at room temperature**.

Centrifuge and Place on the magnetic holder for 1 min.

Transfer **16 µL** of the eluate (containing the amplified cDNA) to the adjacent well

End point : Possibility of storing tubes at -20°C for a long time

cDNA QC

Quantity: Qubit Assay
Quality: Fragment Analyzer

Sample profile of the resulting fragment analyzer for human cell line cDNA

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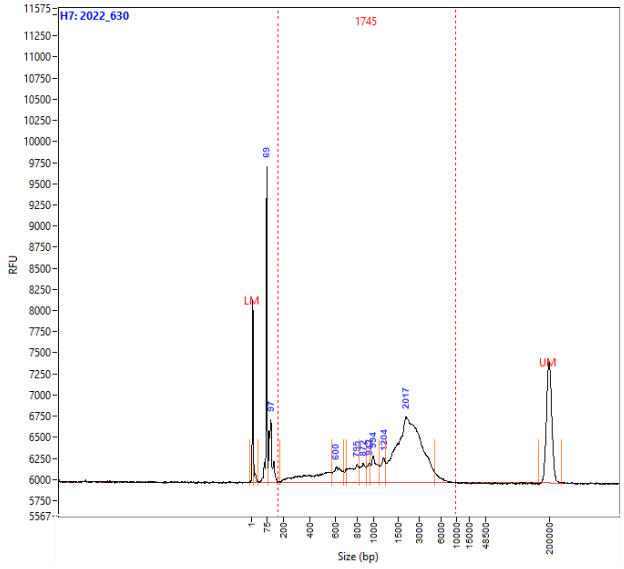

II. End-Prep +purif 1X

Reagents

- NEBNext Ultra II End repair/dA-tailing Module (NEB, cat # E7546)
- Agencourt AMPure XP beads (Beckman Coulter™ cat # A63881)
- Eau Nuclease-free
- Absolute Ethanol

Prepare 70% ethanol with nuclease free water (can be frozen for later use)
Bring the balls to room temperature for 15 minutes
Thaw Ultra End-prep Reaction Buffer Reagents at room temperature before putting them in ice, take the enzyme out into ice at the last moment

Start the **END-PREP program** on the PCR machine for preheating:

5min at 20°C
5min at 65°C
Cool to 4°C

From **20fmol max of cDNA** in a 96-well plate to add the following reagents:

Volume Responsive	
Max 20 fmol de cDNA	qsp 50µL H20
Ultra-End-prep reaction Buffer	7µl
Ultra II End-prep enzyme Mix	3µl
Total 60 µl	

Mix gently with a pipette and centrifuge
Put in the PCR machine and start the **END-PREP program**
Centrifuger

Proceed to the purification step:

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Properly resuspend the AMPure XP beads with the vortex
Add **60ul of AMPure XP Beads (1X)**
Mix by pipetting 10X (final volume of 120 µl)
Leave **5min to RT** on the bench
Centrifuger
Put on the magnetic holder **for 2 min**
Removing the supernatant
Add 200µl of just prepared 70% ethanol and leave for **30 sec**
Remove 70% ethanol with a pipette
Repeat the wash a second time
Thoroughly remove residual ethanol with a 10µL pipette
Let dry for 2 minutes, but do not let the base crack
Remove the plate from the holder and add **16 µl of H2O,** mix well
Incubate at RT on the bench **2min**
Put on the magnetic holder until the liquid becomes clear
Recover 15µl from the eluate

End point : it is possible to store samples at 4°C at night

III. PCA ligation + purif 0.6

Material

- ONT Kit EXP-PCA001 PCR Adapter (PCA)
- Agencourt AMPure XP Beads (Beckman CoulterTM, cat # A63881)
- NEB Blunt/TA Ligase Master Mix (NEB, cat #M0367)
- Eau Nuclease-free
- Absolute Ethanol

Prepare 70% ethanol with nuclease free water (can be frozen for later use)
Thaw reagents in ice except enzymes that are not frozen
Bring the balls to room temperature for at least 15 minutes

Add the reagents in the order of the table. **Between each addition, mix well 10-20 times with the pipette.**

Volume Responsive	
End-prepped cDNA	15 µl
PCR Adapter (PCA) 10 µl	
Blunt/TA Ligase Master Mix	25 µl
Total 50 µl	

Mix well with the pipette and centrifuge briefly
Incubate for 10 min at room temperature

Proceed to the purification step:
Properly resuspend the AMPure XP beads with the vortex
Add 30 µl of resuspended beads to do a 0.6X wash and make 10 round trips with the pipette (final volume of 80 µl)
Incubate for 5 min at room temperature

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Centrifuge and put on the magnetic plate until the balls form a base.
Remove the sunageant
Add 200µl of just prepared 70% ethanol and leave for **30 sec**
Remove 70% ethanol with a pipette
Repeat the wash a second time
Thoroughly remove residual ethanol with a 10µL pipette
Let dry for 30 seconds, but do not let the base crack
Remove the tube from the magnetic holder and **resuspend in 23.5 µL of** nuclease-free water
Incubate for 2 min at room temperature
Centrifuge and put on magnetic media
Allow the beads to form a pellet until the eluate is clear
Recover 22 µL of Eluate

Quantify with 2 µL at the Qubit

For the concentration calculation, take the size of the cDNA obtained after amplification of the RNA. There is no need for a new migration in electrophoresis.

Take the next step

IV. PCR addition of barcodes + 0.6X purif

Material

- ONT Kit SQK-PCB114 Barcode Primers (BP01-24)
- Elution Buffer (EB) of ONT Kit SQK-PCB114
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- LongAmp Hot Start Taq 2X Master Mix (NEB, M0533S)
- Agencourt AMPure XP beads de Beckman
- Eau Nuclease-free
- Absolute Ethanol

Prepare 70% ethanol with nuclease free water (can be frozen for later use)
Thaw Barcode Primers (BP01-24) at room temperature and LongAmp Hot Start in ice

Take **2 fmol of cDNA** from the previous step of PCA ligation
Mix the reagents in a PCR plate:

Reagent		Volume
PCR PCA Ligated cDNA (2fmol):		**xµl**
Unique Barcode Primer (BP01-24)		1,5 µl
Nuclease-free water	qsp	25 µl
2x LongAmp Hot Start Taq Master Mix		25 µl
Total 50 µL		

Launch the following program LIG-Ampl

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Step	Temperature	Time	Cycle
Initial denaturation	95°C	3 min	1
Denaturation	95°C	15 sec	18
Annealing	**62°C**	15 sec	18
Extension	**65°C**	**17 min**	**18**
Final extension	65°C	6 min	1
Hold 4°C ∞			

The program lasts at least 5 hours and can be launched on the night

Bring the beads and the Elution Buffer (EB) back to room temperature for at least 15 minutes

In this protocol we do not use Exonuclease 1

Proceed to the purification step:
Properly resuspend the AMPure XP beads with the vortex
Add 30 µl of resuspended beads to do a 0.6X wash and make 10 round trips with the pipette (final volume of 80 µl)
Incubate for 5 min at room temperature
Centrifuge and put on the magnetic plate until the balls form a base.
Remove the sunageant
Add 200µl of just prepared 70% ethanol and leave for **30 sec**
Remove 70% ethanol with a pipette
Repeat the wash a second time
Thoroughly remove residual ethanol with a 10µL pipette
Let dry for 30 seconds, but do not let the base crack
Remove the tube from the magnetic holder and **resuspend in 51.5 µl of EB buffer**
Incubate for 10 min at room temperature
Centrifuge and put on magnetic media
Allow the beads to form a pellet until the eluate is clear
Recover 50 µL of Eluate

Repeat a second purification cycle:
Add 30 µl of resuspended beads to do a 0.6X wash and make 10 round trips with the pipette (final volume of 80 µl)
Incubate for 5 min at room temperature
Centrifuge and put on the magnetic plate until the balls form a base.
Remove the sunageant
Add 200µl of just prepared 70% ethanol and leave for **30 sec**
Remove 70% ethanol with a pipette
Repeat the wash a second time
Thoroughly remove residual ethanol with a 10µL pipette
Let dry for 30 seconds, but do not let the base crack
Remove the tube from the magnetic holder and **resuspend in 14.5 µL of EB buffer**
Incubate for 10 min at room temperature
Centrifuge and put on magnetic media
Allow the beads to form a pellet until the eluate is clear
Recover 13 µL of Eluate

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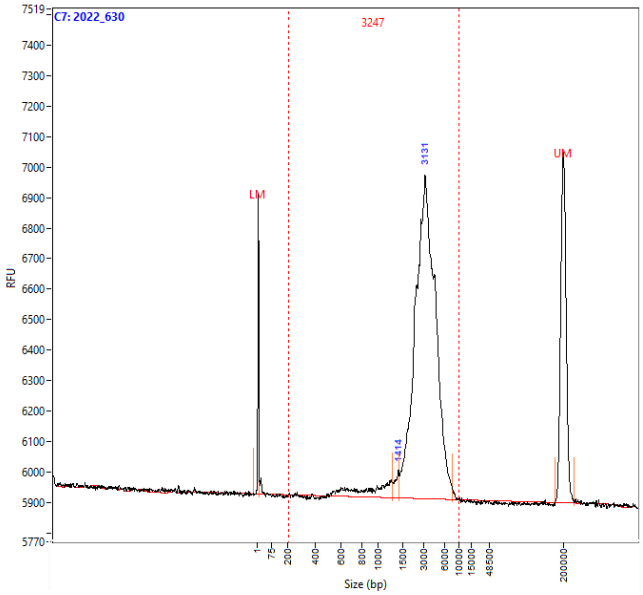
cDNA QC

Quantity: Qubit assay with (2 µl)
Quality: Fragment Analyzer (1 µl if little material otherwise prefer 2 µl)

Sample profile of the resulting fragment analyzer for human cell line cDNA



Attention Storage:

We recommend storing the libraries in Eppendorf DNA LoBind tubes **at 4°C for short-term storage until the end of the project** or repeated use, e.g. to recharge the FlowCells between washes.

For **long-term storage** , we recommend storing the libraries **at -80°C** in Eppendorf DNA LoBind tubes

V. Attaching RAP Adapters (Chemistry 14)

Material

- amplified and barcoded cDNA
- Rapid Adapter (RA) of the SQK-PCB114 kit
- Elution Buffer (EB) du kit SQK-PCB114
- SQK-PCB114 Kit Buffer Adapter (ADB)

Multiplexer **50 fmol** of cDNA amplified and barcoded in Elution Buffer (EB) under the following conditions:

-in a final volume of **11 µl** for sequencing on **MinIon**

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-in a final volume of **31 µl** for sequencing on **PromethIon**

Briefly centrifuge the Rapid Adapter (RA) tube as well as the Adapter Buffer (ADB) tube. These tubes are not frozen. Mix them with the pipette, remove the necessary volume and then put them back at -20°C.

Dilute the **Rapid Adapter (RA) reagent as follows:**

Volume Responsive
Rapid Adapter (RA) 1.5 µl
Adapter Buffer (ADB) 3.5 µl
Total 5 µl

Mix well by pipetting and centrifuging

Add 1 µL of the just-diluted Rapid Adapter (RA) to the **50 fmol mix of** amplified and barcoded cDNA library in a final volume of **12 µL** for **the MinION** or **32 µL** for **the PromethION**

Mix well by pipetting and centrifuging
Incubate for 5 min at room temperature

The library is now ready to be sequenced in the Fow Cell and must be **kept in ice until loading.**
Follow the MinION or PromethION protocol for chemistry 14

VI. Sequencing

Follow the MinION or PromethION protocol for chemistry 14

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