Library Preparation Manual

Version 190219

SPATIAL TRANSCRIPTOMICS

For Research Use Only

Table Of Contents

Revision History

Revised Part	Date	Revision
Required Consumables	2016-09-09	Adding DynaMag™-2 Magnet as magnetic rack for tubes
7, 8, 9, 10, 12, 13	2016-09-09	Specify DynaMag™-2 Magnet as magnetic rack for tubes
Required Consumables	2016-10-04	Updated concentrations for PCR InPE 1 (50 µM) and PCR InPE 2 (1 µM)
Preparing Stock Reagents	2016-10-04	Updated concentrations for PCR InPE 1 (50 µM) and PCR InPE 2 (1 µM)
All parts	2016-10-17	Minor updates of instructions and reagent amounts/descriptions
All parts	2016-11-15	Minor updates and more specified description of some reagents.
12	2016-12-19	Update reagent amount in Purification
All parts	2017-02-10	A different slide module is suggested to be used during the protocol
Preparing Stock Reagents and Buffers	2017-07-11	Update recipe for Tris / Acetic Acid Buffer
All parts	2018-03-29	Minor updates throughout
Pages 33 and 35	2018-05-15	Added missing concentrations
Appendix	2018-05-29	Added new slide modules
Pages 11, 12, 27, 29	2018-06-11	Slide schematic and second strand synthesis
Before You Start. Permeabilisation.Required Equipment.	2019-02-19	ProPlate hybridization cassettes recommended for single-use only, and minor updates.

Introduction

Library Preparation

The Library Preparation (**LP**) protocol generates spatially barcoded ready-to-load sequencing libraries from fresh frozen tissue sections. We recommended performing a Tissue Optimisation (**TO**) experiment prior to beginning LP, in order to optimise the protocol for your tissue of interest.

This Protocol only works with fresh frozen tissue.

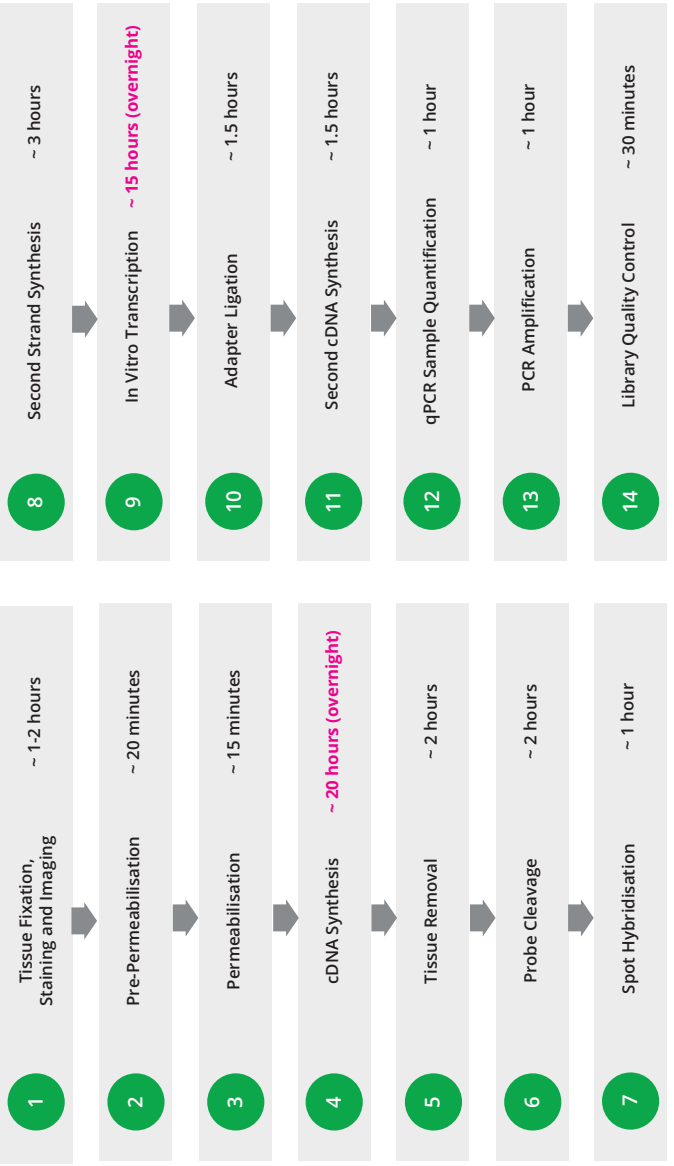
The LP glass slide has six square subregions. Each subregion contains an array of spotted surface probes that capture mRNA and serve as primers for reverse transcription. The millions of surface probes in an individual spot share a unique positional barcode that allows them to be distinguished from the surface probes in other spots. Each array consists of 1007 of these spots.

A tissue section is placed on each array, fixed, stained with Hematoxylin and Eosin (H&E) and imaged. Next, reagents are added to permeabilise the cells and allow the mRNA from the tissue sections to hybridize to the adjacent surface probes on the glass surface. A cDNA synthesis reaction is then performed. Once cDNA synthesis is complete, the tissue needs to be removed from the slide (a critical step optimised via a TO experiment).

Following tissue removal the surface probes/cDNA, with attached mRNA, are cleaved from the slide surface. The probes from each array are collected in separate tubes. Finally, the cleaved probes are used to prepare ready-to-sequence libraries.

The first steps of the LP protocol are very similiar to the TO protocol. Therefore the parameters determined in the TO experiment can be transferred to the LP protocol.

Workflow



General instructions

Before You Start

- **Read this manual thoroughly.**
- **When the LP label faces up the active surface of the slide faces up.**
- **Never touch the active surface of the slide!**
- **The LP slide is placed into the slide module (microarray hybridization cassette) for pre-permeabilisation -NOT BEFORE.**
- **Grace Biolabs ProPlate slide modules (RD482441) are disposable. We do not recommend reuse.**
- **Perform the protocol without interruption unless otherwise stated. Safe stopping points are indicated throughout the protocol.**
- **All reagents should be vortexed and spun down before pipetting if not otherwise noted.**
- **Prepare stock solutions and buffers (page 45) before beginning your LP experiment.**

We recommend performing a TO experiment prior to beginning LP in order to optimise the protocol for your tissue of interest. Parameters that can be changed for different tissue types are highlighted in orange throughout the text, as shown here: **7 minutes.**

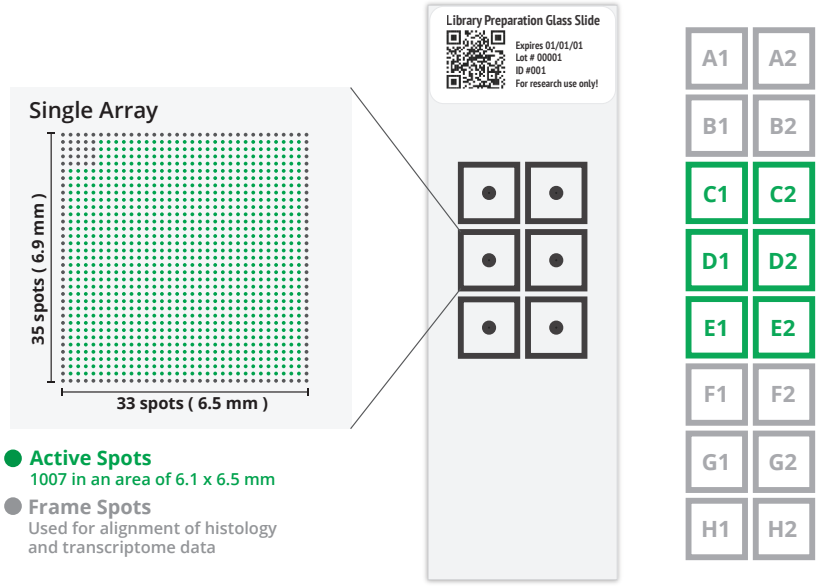
During some incubation steps, reagents for the subsequent step in the protocol need to be prepared. These preparatory steps are highlighted with **purple text**.

*Please read carefully all **M**aterial **S**afety **D**ata **S**heets provided by the individual reagent suppliers carefully. By using this protocol you agree to the terms and conditions that can be found under* **https://www.spatialtranscriptomics.com/terms-and-conditions/**

Experimental Design

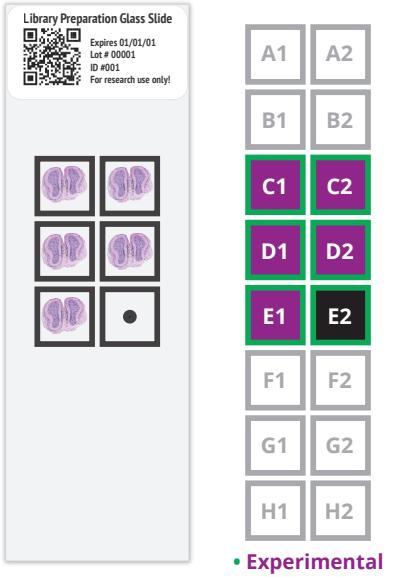
• The LP slides have six square subregions that contain arrays of spotted surface probes with unique positional barcodes. The six subregions that house the arrays are demarcated by black frames printed on the back of the slide.

• The slide module that is used to create individual reaction wells generates two columns (1-2) of eight rows (A-H). The active subregions on LP slides correspond to rows C, D, and E, as indicated in the picture below.



• We recommended placing tissue sections on five of the arrays for full Spatial Transcriptomics analysis, and using the sixth array for a positive control

• If the user does not wish to perform a positive control, then tissue sections can be placed on all six arrays.



• **Experimental**
• **Positive Control**

Positive Control

• A positive control is used to confirm successful cDNA synthesis conditions. **Do not** place a tissue section on this subregion. A positive control well is treated with control RNA and cDNA master mix only.

• In order for the positive control to be as accurate as possible, the RNA used should be fragmented to a fragment size of approximately 300 bp. We recommend using a commercially available kit for RNA fragmentation.

RNA Quality Requirements

Agilent Bioanalyzer RNA Pico Kit
RNA Extraction kit

• In order to ensure the best possible results we recommend using tissue with a **RIN (RNA Integrity Number) value of 7 or higher**.

• To evaluate tissue RIN we recommend collecting a sample containing at least 8 tissue sections (10 µm thick) for testing. We also recommend the use of a commercially available RNA extraction kit and 2100 Bioanalyzer analysis to determine the RIN value.

• To achieve a representative RIN please ensure that the tissue does not thaw prior to RNA extraction. One should, for example, pre-cool the extration tube into which the tissue sections are placed, and handle the extraction tube with forceps.

Tissue Handling & Sectioning

- Store fresh frozen tissue at -80°C and avoid thawing.

- If tissue was frozen without Optimal Cutting Temperature (**OCT**) embedding medium, it should be embedded in OCT prior to sectioning. Cool the OCT close to the point where it hardens before embedding.

- A single array of spots on an LP slide measures 6.5mm x 6.9mm. Tissue sections should be no larger than 6.3 x 6.7mm in order to fit within the frame spots (visible frame spots are required for data alignment).

- Tissue + OCT should fit within a 10x10mm square so that sections do not overlap (the OCT is dissolved and washed away during fixation and staining).

- Tissue should be sectioned with a cryostat.

A Video Demonstration of the Sectioning process can be found at: https://vimeo.com/154600991

- Pre-cool your LP slide in the cryostat.

- We recommend a tissue section thickness of 5-16 µm (see page 44 for tissue specific suggestions).

IMPORTANT: Flatten the tissue section carefully. Make sure not to touch the tissue itself but rather touch the surrounding OCT. Place a finger on the backside of a square on the TO slide for several seconds in order to warm the glass. Once the targeted area of the slide is heated, the tissue should attach automatically when the slide is held close to it.

- **Once tissue sections have been placed on the LP slide, the slide can either be stored at -80 °C for up to 7 days or the LP experiment can be started immediately.**

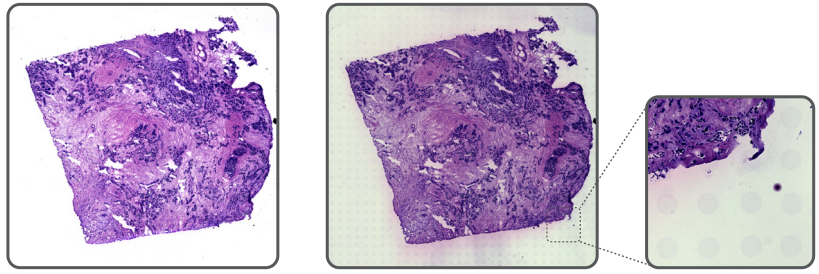
Brightfield Scanning

A brightfield imaging system is required to capture the H&E stainings.

In order to generate high resolution images of the stained tissue sections in a reasonable timeframe we recommend low magnification, high numerical aperture (NA), objectives e.g. 10x NA 0.45, 20x NA 0.5, 20x NA 0.75.

At these magnifications many captured images (tiles) will be needed to reconstruct your entire tissue section. These are usually captured with the aid of a motorized scanning microscope stage.

The H&E staining results in lightly stained array spots. It is important to capture the stained spots in your image to allow later data alignment. To ensure that the stained spots of the array are visible it is necessary to **avoid overexposing the image**. In fact a slight underexposure often yields the best results (see below). i.e. your camera exposure time may need to be shorter than suggested by the "auto-expose" function in your imaging software.



Human Breast cancer tissue on a Library Preparation Slide

Reduced exposure

Visible Spots

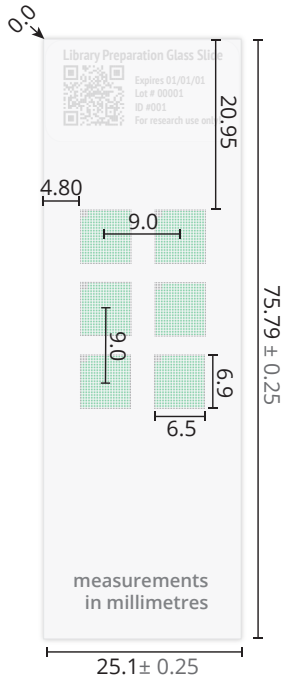
It is important to image the arrays of spots in their entirety.
(see page 12 for schematic).

Fluorescent Scanning

In order to properly align the H&E images with the spatially resolved transcriptome data from each spot on an array, a fluorescent image of all spots has to be created at the end of the experimet. These fluorescent spots are then aligned with the Hematoxylin stained spots in the brightfield image.

The fluorescent dye used in the protocol is Cyanine-3. Cyanine-3 has an excitation maximum of approximately 550nm and an emission maxima of approximately 570nm.

Below is a schematic of the LP slide. Six 6.5mm x 6.9mm arrays, with a distance of 9mm between centre points. **It is important to image the arrays in their entirety.**



Library Prepration Protocol

1.Tissue Fixation, Staining & Imaging

Formaldehyde solution, 36.5-38%	Eosin Y (aqueous)
1XPBS	Tris/Acetic acid buffer
Isopropanol (2-Propanol)	85% Glycerol
Mayer's Hematoxylin Solution	HBSS buffer
Bluing Buffer	BSA, 20mg/ml

We recommended optimising the duration of the H&E stain for your tissue type of interest prior to starting the LP protocol.

- **Pre-heat a thermomixer for plates and a thermomixer for tubes to 37°C.**
- **Prepare fresh formaldehyde solution by adding 100 µl formaldahyde to 900 µl PBS.**
- **Mix 5 ml Eosin with 45 ml Tris/Acetic acid buffer in a 50 ml tube to prepare Eosin solution.**
- **Prepare Pre-permeabilisation buffer: 986 µl HBSS + 10 µl BSA. Prehaeat to 37°C.**
- **Prepare 50 ml tube of 80% ethanol.**
- **Prepare 50 ml tube of PBS.**
- **Prepare 50 ml tube of ultrapure (e.g. Milli-Q) water.**
- **Prepare three beakers containing 800 ml of ultrapure water each.**

1.Tissue Fixation, Staining & Imaging

1. Heat the LP slide for **1 minute** at 37°C in a thermomixer.

2. Immediately after heating, place the slide on a flat clean surface inside a laminar hood.

3. Apply 900 µl of formaldehyde solution to the active surface of the glass slide, and ensure that all tissue sections are covered.

4. Incubate at room temperature for **10 minutes**.

5. Decant the formaldehyde solution from the glass slide and immediately wash by slowly, and completely, dipping the slide into a 50 ml tube filled with PBS. Dip the slide in PBS **five times**.

6. Remove excess fluid from the slide by wiping the outer edge with tissue paper. **Do not to touch the active surface of the glass slide!**

7. Return the slide to a flat surface and pipette 500 µl isopropanol on to it, making sure that all squares are covered. Incubate for **1 minute**.

8. Remove excess fluid from the slide by wiping the outer edge with tissue paper. Let the slide **air-dry**.

9. When completely dry, pipette 900 µl of Mayer's hematoxylin on to the slide, making sure all squares are covered. Incubate for **7 minutes**.

10. Dip the slide in a 50 ml conical centrifuge tube containing ultrapure water. Repeat **5 times**.

11. Dip the slide in a beaker containing 800 ml ultrapure water. Repeat **15 times**.

12. Taking a fresh beaker, dip the slide in 800 ml ultrapure water. Repeat **15 times**. Let most of the excess water drip off and wipe the back of the slide with tissue paper.

13. Pipette 900 µl Bluing Buffer on to the slide so that it covers all 6 squares. Incubate at room temperature for **2 minutes**.

1. Dip the slide in a beaker of ultrapure water. Repeat **5 times**.

2. For counterstaining dip the glass slide for **1 min** in a 50 ml centrifuge tube containing the freshly prepared Eosion solution.

3. Dip the slide in a beaker of ultrapure water. Repeat **10-15 times**.

4. **Air dry** the glass slide until the tissue sections are completely dry.

5. Incubate the slide for **5 minutes** at 37°C.

6. Remove the black squares from the back of the slide with an ethanol soaked tissue.

7. Carefully pipette 200 µl of 85% glycerol on to the tissue sections and cover with a coverglass. Image the sections.

8. Remove the cover glass by holding the glass slide and dipping it in a beaker of ultrapure water. Gently move the glass slide up and down and let the cover glass detach under its own weight. Once the cover glass has detached, dip the slide in the water three times.

9. Dip the glass slide swiftly in 80% ethanol in order to remove any remaining glycerol.

10. Air dry at room temperature.

11. Warm the slide for **1 minute** at 37°C.

- **Proceed directly to Pre-Permeabilisation.**

2. Pre-Permeabilisation

Collagenase, 50U/µl
Pepsin (0.1%) in HCl aliquot

1. Prepare Pre-permeabilisation mix by adding 4 µl collagenase to the Pre-Permeabilisation buffer. Invert carefully, spin down quickly and put back at 37°C until use. Do not vortex!

2. **Place the slide in the slide module**. Make sure to tighten the screws.

3. Add 70 µl of the Collagenase mix to each of the wells containing a tissue section.

Whenever pipetting into wells containing tissue sections be careful not to pipette directly onto the tissue or to touch the tissue with the pipette tip. Rather pipette down slowly at the edge of the well.

4. Cover all sample wells with a plastic seal to avoid evaporation.

5. Incubate at 37°C for **20 minutes** (no shake) in a thermomixer.

During the incubation, prepare reagents needed for the cDNA synthesis (See page 18). But **do not** add SuperScript III or RNaseOUT at this point!

When 10 min of the Pre-Permabilization incubation remain, pre-heat one aliquot of Pepsin to 37°C.

6. When the incubation is finished: carefully remove the seal and Pre-Permeabilisation mix from all sample wells by slowly pipetting.

7. Wash each well carefully by slowly adding 100 µl 0.1x SSC.

3. Permeabilisation

Pepsin (0.1%) in HCl aliquot

The optimal duration of the permeabilisation step for your tissue of interest should have been established with a prior TO experiment.

1. Exchange the SSC in each well for 70 µl of Pepsin, and incubate at 37°C for the optimized duration identified with a TO experiment.

2. When the incubation is finished carefully remove the Pepsin mix by pipetting slowly.

3. Wash all experimental wells carefully by slowly adding 100µl 0.1x SSC.

- **Proceed directly to cDNA synthesis**

4. cDNA Synthesis

First Strand Buffer (5X)	Actinomycin D (5 mg/ml in DMSO, as previosly aliquoted)
0.1 M DTT	BSA (20 mg/ml), molecular biology grade
dNTP (10 mM each)	SuperScript® III Reverse Transcriptase
Water (RNase & DNase free)	RNaseOUT™ Recombinat Ribonuclease Inhibitor

- Prepare the cDNA synthesis buffer mix in a 1.5 ml tube as follows:

First Strand Buffer (5X)	96 µl
0.1 M DTT	24 µl
Water (DNase/RNase free)	255.2 µl
Actinomycin D	4.8 µl
BSA (20 mg/ml)	4.5 µl
dNTP (10 mM each)	24 µl

Mix well by pipetting up and down several times and preheat to 42 °C.

1. Add 48 µl SuperScript® III and 24 µl of RNAseOUT™ to the cDNA synthesis buffer mix tube. Mix by pipetting up and down carefully. This tube now contains the finished cDNA synthesis master mix. Put the tube back at 42°C.

2. Slowly remove the 0.1x SSC buffer from all the wells.

3. Add 75 µl of cDNA synthesis master mix to all five (or six) wells. Avoid bubbles in the wells by not releasing the last few microliters from the pipette tip.

4. Optional: Add 1-2 µg of the positive control RNA to 70 µl of the left cDNA Synthesis master mix and mix by pipetting up and down slowly. Add 70 µl of this mixture to the positive control well.

5. Cover all wells with a plastic seal.

6. Incubate at 42°C for **18-20 hours** (no shake).

5. Tissue Removal

There is a choice between two tissue removal procedures:

Option 1: For less fibrous, fatty tissue like brain. **Proteinase K** treatment.

Option 2: For more fibrous tissue. **β-mercaptoethanol** followed by **Proteinase K**.

Please use the conditions you have established for your tissue in a prior TO experiment.

Option 1: Proteinase K tissue removal

Proteinase K
Buffer PKD

1. Mix 420 µl PKD buffer with 60 µl proteinase K, by pipetting (do not vortex) and pre-heat to 56°C.
2. Remove the seal from the slide module and carefully remove the cDNA Synthesis mixture from the wells by pipetting slowly.
3. Wash each well by adding 100 µl 0.1xSSC and then removing it slowly.
4. Add 70 µl proteinase K tissue removal mixture to each sample well. Seal all wells with a plastic seal.
5. Incubate the glass slide in a thermomixer at 56°C for **1hour** with interval mixing (e.g. 300 rpm, 15 second shake, 15 second rest).
6. Remove the plastic seal and remove the proteinase K from the wells.
7. Remove the glass slide from the slide module.
8. Pour 100 ml SSC/SDS buffer preheated to 50 °C into a wash pan.
9. Place the glass slide on the bottom of the wash pan with the active surface facing upwards so that the glass slide is covered by the buffer.
10. Incubate for **10 minutes** at 50°C while shaking (300 rpm).
11. During the incubation: Prepare a second wash pan with 100 ml 0.2x SSC and a third wash pan with 100 ml 0.1x SSC.
12. Move the glass slide to the second wash pan filled with 100 ml 0.2x SSC at room temperature and incubate for **1 minute** while shaking (300 rpm).
13. Move the glass slide to the third wash pan filled with 0.1x SSC at room temperature and incubate for **1 minute** while shaking (300 rpm).
14. Spin the glass slide in a centrifuge for glass slides for 5 seconds or until dry. In case no glass slide centrifuge is available, the slide can be dried at room temperature.

5. Tissue Removal

Option 2: β-mercaptoethanol followed by Proteinase K

β-mercaptoethanol	Buffer RLT or RLT Plus
Proteinase K	Buffer PKD

In a fume hood:

Prepare **1x** β-mercaptoethanol tissue removal reagent by mixing **990 µl** RLT buffer with **10 µl** β-mercaptoethanol in a 1.5ml eppendorf tube.

Pre-heat the β-mercaptoethanol mix to 56°C for at least 5 min.

1. Remove the seal from the hybridization cassette and carefully remove all cDNA Synthesis Reagent mixture from the wells by pipetting slowly.

2. Wash each well carefully with 100 µl 0.1xSSC.

3. Add around 70 µl of the β-mercaptoethanol mix to each active well; try to avoid bubbles in the wells. Cover wells with a plate sealer.

4. Incubate at 56°C with 300 rpm shake for the following time depending on tissue type:

Tissue Type	**β-mercaptoethanol incubation time**
less fibrous tissues	**1 h**
fibrous tissues (such as human heart)	**1 hour 30 minutes**

5. When ~5 min of the incubation remains, mix 420 µl PKD buffer with 60 µl proteinase K by pipetting (do not vortex) and pre-heat to 56°C.

6. Remove the β-mercaptoethanol mix from each well by pipetting.

7. Wash each well by adding 100 µl 0.1xSSC and then removing it slowly.

8. Add 70 µl proteinase K tissue removal mixture to each sample well. Seal all wells with a plastic seal.

9. Incubate the glass slide in a thermomixer at 56°C for **1 hour** with interval mixing (e.g. 300 rpm, 15 second shake, 15 second rest).

10. Remove the plastic seal and remove the proteinase K tissue removal mixture from the wells.

11. Remove the glass slide from the slide module.

12. Pour 100ml SSC/SDS buffer preheated to 50 °C into a wash pan.

13. Place the glass slide on the bottom of the wash pan with the active surface facing upwards so that the glass slide is covered by the buffer.

14. Incubate for **10 minutes** at 50°C while shaking (300 rpm).

15. During the incubation: Prepare a second wash pan with 100 ml 0.2x SSC and a third wash pan with 100 ml 0.1x SSC.

16. Move the glass slide to the second wash pan filled with 100 ml 0.2x SSC at room temperature and incubate for **1 minute** while shaking (300 rpm).

17. Move the glass slide to the third wash pan filled with 0.1x SSC at room temperature and incubate for **1 minute** while shaking (300 rpm).

18. Spin the glass slide in a centrifuge for glass slides for 5 seconds or until dry. In case no glass slide centrifuge is available, the slide can be dried at room temperature.

- **Proceed immediately to Probe Cleavage**

6. Probe Cleavage

Second Strand Buffer (5X)	USER™ Enzyme
dNTP (10 mM each)	BSA (20 mg/ml)

1. Place the slide in a slide module.

2. Prepare cleavage buffer by adding following reagents to a 1.5 ml tube:

Water (DNase/RNase free)	320 µl
Second Strand Buffer (5X)	104 µl
dNTP (10 mM each)	4.2 µl
BSA (20 mg/ml)	4.8 µl

3. Pre-heat the cleavage buffer to 37 °C. Do not vortex but spin down the tube.

4. Add 48 µl USER™ Enzyme to the cleavage buffer tube. Mix well by pipetting up and down at least 10 times. Pre-heat tube to 37°C again.

5. Add 70 µl of the cleavage buffer/USER™ Enzyme solution to all 6 wells, avoiding bubbles. If bubbles are visibile, remove them with a pipette tip as bubbles can lead to a less efficient probe cleavage on the surrounding surface.

6. Cover wells with a plate sealer and incubate at 37°C for **2 hours** (300 rpm shake for 15 sec with 15 sec rest in between).

7. After the incubation: collect 65µl from each well into 0.2ml low DNA binding tubes. Quickly spin down tubes and keep on ice.

8. The glass slide can be kept in the slide module (covered with a plate sealer) for the spot hybridization.

9. OPTIONAL: Spot Hybridisation and Scanning can be performed during the Second Strand Synthesis incubation.

Pause point: Cleaved probes can be stored at -20°C after this step.

7. Spot Hybridization

1X Phosphate buffered saline (PBS)	SlowFade® Gold Antifade Mountant
Cyanine-3 A Probe (10 µM)	Cyanine-3 Frame Probe (10 µM)

In order to determine the exact location and quality of each of the 1007 spots the fluorescent Cyanine-3 A probe is hybridized to the 5' ends of the surface probes that remain attached to the glass folllowing cleavage. The frame spots, which yield a strong signal for data alignment, are similarly targetted with Cyanine-3 Frame Probe.

1. Add the following reagents to a tube, mix well by vortexing:

1X Phosphate buffered saline (PBS)	960 µl
Cyanine-3 A Probe (10 µM)	20 µl
Cyanine-3 Frame Probe (10 µM)	20 µl

2. Add 75 µl of this hybridisation solution to each well, avoiding bubbles. The remaining hybridisation solution can be stored at -20°C.

3. Incubate at room temperature for **10 min**.

4. At the end of the incubation, pre-heat ~100ml SSC/SDS buffer (2xSSC, 0.1% SDS) to 50°C.

5. Pour pre-heated SSC/SDS buffer into a wash pan and remove the hybridisation solution from each well by pipetting.

6. Detach slide from the slide module.

7. Place the glass slide on the bottom of the wash pan with the active surface facing upwards so that the glass slide is covered by the buffer.

8. Incubate for **10 min** at 50°C with shaking (300 rpm).

9. Move the glass slide to a second wash pan filled with 100 ml 0.2x SSC at room temperature and incubate for **1 min** with shaking (300 rpm).

10. Move the glass slide to a third wash pan filled with 0.1x SSC at room temperature and incubate for **1 min** with shaking (300 rpm).

11. Spin the glass slide in a centrifuge for glass slides for 5 seconds or until dry. In case no glass slide centrifuge is available, the slide can be dried at room temperature.

12. Apply SlowFade® Gold Antifade Mountant in between the arrays and cover with a cover slip. Make sure the mountant medium covers all the wells and that no bubbles are visible.

13. Image. We recommend using the same magnification and scan area as for the bright field images.

8. Second Strand Synthesis

First Strand Buffer (5X)	500 mM EDTA
DNA polymerase I (10 U/µL)	Agencourt RNAClean XP beads
Ribonuclease H (2 U/µL)	80% ethanol
T4 DNA polymerase (3 U/µl)	NTP mix from MEGAscript® T7 Transcription Kit

Bring the Agencourt RNAClean XP beads to room temperature at least 30 min before use and vortex thoroughly before adding to the tubes.

Pool the four aliquots of NTP solution from the MEGAscript® T7 Transcription Kit into one tube and mix well by pipetting up and down at least 10 times.

Mix 45 µl of the NTP pool with 39 µl of water (RNase/DNase free), and place on ice.

1. Set a thermal cycler to 16°C (with lid at 16°C or without the lid closed).

2. Prepare a tube with the following Invitrogen reagents, mix well by pipetting (keep cold):

First Strand Buffer (5X)	20 µl
DNA polymerase I (10 U/µL)	14 µl
Ribonuclease H (2 U/µL)	3.5 µl

3. Add 5 µl to each of the six sample tubes, mix well by pipetting (keep cold). **Attention! Do not let the sample temperature rise above 16°C!**

4. Remove bubbles and spin down. Incubate at 16 °C for **2 hours** (thermal cycler with lid at 16°C or without the lid closed). Then cool the samples to 4 °C.

OPTIONAL: During this Incubation it its possible to perform the Spot Hybridisation and Scanning step.

5. Once the incubation is finished, place tubes on ice.

6. Dilute 500 mM EDTA to 80 mM and keep on ice:

Water (DNase/RNase free)	158 µl
500mM EDTA	30 µl

7. Add 5µl NEB T4 DNA polymerase (3 U/µl) to each sample tube. Mix well by pipetting (keep cold). **Attention! Never let the sample temperature rise above 16°C!**

8. Remove bubbles with a pipette tip and spin down.

9. Incubate at 16°C for **20 min** (thermal cycler with lid at 16°C or without the lid closed). Place tubes on ice directly after incubation and immediately proceed to the next step.

10. Add 25 µl cold 80 mM EDTA to each of the six tubes, mix well by pipetting.

11. Place tubes on ice and proceed to the next step immediately.

cDNA Purification

12. Add 100 µl Agencourt RNAClean XP beads to each of six 1.5 ml low DNA binding tubes.

13. Mix 100 µl sample with 100 µl beads by pipetting up and down 10 times and incubate for **15 min** at RT for binding.

14. Place the tubes in a DynaMag™-2 Magnet and incubate at RT for **5 min** in order for the beads to separate. **Do not remove the tubes from the magnetic rack until step 18**.

15. Remove buffer by slow pipetting. **Do not touch the beads**.

16. Add 1000 µl 80% ethanol to each tube. After 30 seconds, slowly remove the ethanol by pipetting. **Do not touch the beads**.

17. Repeat step 16 twice more, for a total of three ethanol treatments.

18. Let air dry, with the lids open, until you see the first signs of cracking. **Do not over dry the samples!**

19. Suspend beads in 13 µl of the NTP/water mix. In this step you have to touch the beads with the pipette tip.

20. Incubate for **2 min** at room temperature (not on magnetic rack).

21. Place tubes on magnetic rack for **5 min**.

9. In Vitro Transcription

MEGAscript® T7 Transcription Kit	80% ethanol
SUPERase In™ RNase Inhibitor (20 U/µL)	Agilent RNA 6000 Pico Kit
Agencourt RNAClean XP beads	

Attention! Thaw the 10X Reaction buffer from the MEGAscript® T7 Transcription Kit well before making the master mix and vortex it properly to make sure no precipitation is visible in the tube.

1. Prepare a tube, at room temperature, with the following reagents from the MEGAscript® T7 Transcription Kit, mix well by pipetting:

10X Reaction Buffer	12 µl
T7 Enzyme Mix	12 µl
SUPERase In™ RNase Inhibitor (20 U/µL)	6 µl

2. Add 4 µl of this reaction mix to each of six 0.2 ml low DNA binding tubes.

3. Add 12 µl of eluted cDNA to 4 µl of reaction mix, and mix by pipetting.

4. Remove bubbles and spin down.

5. Incubate at 37°C for **14 hours** followed by 4°C for ∞ (thermal cycler with 70°C heated lid).
   Attention! This step should be carried out overnight to avoid the storage of aRNA at 4°C for long periods of time.

Pause Point: Samples can be stored at -80 °C after this step.

aRNA Purification

Bring the Agencourt RNA Clean XP beads to RT at least 30 min before use and vortex thoroughly before adding to the tubes.

6. Add 54 µl Agencourt RNAClean XP beads to each of six new 1.5 ml low DNA binding tubes.

7. Add 14 µl water (DNase/RNase free) to each sample tube.

8. Mix 30 µl sample with 54 µl beads by pipetting up and down 10 times and incubate for **15 min** in RT for binding.

9. Place the tubes in a DynaMag™-2 Magnet and incubate at RT for **5 min** in order for the beads to separate. Do not remove the tubes from the magnetic rack until step 14.

10. Remove buffer by slow pipetting; make sure not to touch the beads.

11. Add 1000 µl 80% ethanol to each tube. After **30 seconds**, slowly remove the ethanol by pipetting. Make sure not to touch the beads.

12. Repeat step 11 twice more, for a total of three ethanol treatments.

13. Let air dry, with the lids open, until you see the first signs of cracking.
    Do not over dry the samples!

14. Resuspend the beads in 10 µl water (DNase/RNase free). In this step you have to touch the beads with the pipette tip.

15. Incubate for **2 min** (**not** on magnetic rack).

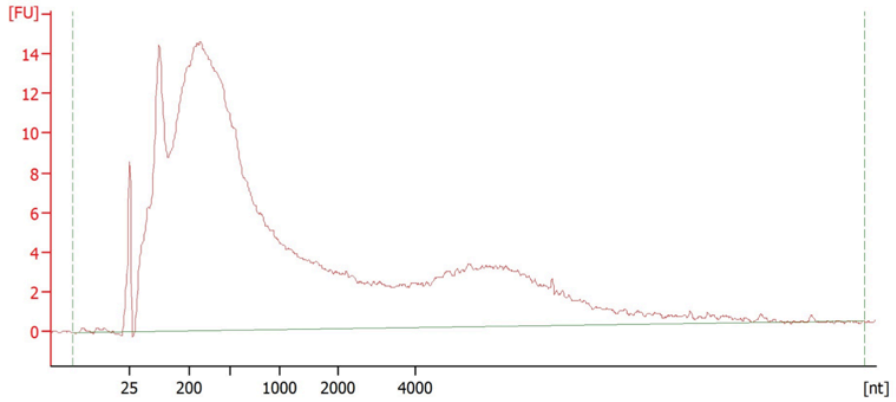
16. Place tubes on magnetic rack for **5 min**.

17. For each sample, transfer 2 µl to a new 1.5 ml tube (used for the Bioanalyzer measurement in step 20).

18. Collect the remaining 8 µl and transfer to a new 0.2 ml low DNA binding tube, and put on ice directly.

19. Heat the 1.5 ml tubes at 70°C for **2 min** and then put directly on ice.

20. Check the quality and the amount of aRNA with a Bioanalyzer by using the Agilent RNA 6000 Pico Kit. Ideally, average length should be between 200 nt and 500 nt. The aRNA should look like this:



Pause Point: Samples can be stored at -80°C after this step.

10. Adapter Ligation

aRNA Ligation Adapter (3 µM)	RNase Inhibitor, Murine
10X T4 RNA Ligase Reaction buffer	Agencourt RNA Clean XP
T4 RNA Ligase 2, truncated	80% ethanol

Bring the Agencourt RNA Clean XP beads to RT at least 30 min before use and vortex thoroughly before adding to the tubes.

1. Set a thermal cycler to 70°C (with heated lid at 105°C).

2. Prepare 0.2ml low DNA binding tubes with the following:
(mix well by pipetting and keep cold)

Purified aRNA	8 µl
aRNA Ligation Adapter (3 µM)	2.5 µl

3. Heat adapter-sample mix at 70°C for **2 min** and then directly put on ice.

4. Set a thermal cycler to 25°C (with lid at 25°C or without the lid closed)

5. Prepare a 1.5 ml tube with the following reagents:
(mix well by pipetting and keep cold):

10X T4 RNA Ligase Reaction buffer	11.3 µl
T4 RNA Ligase 2, truncated	11.3 µl
RNase Inhibitor, Murine	11.3 µl

6. Add 4.5 µl of the ligation mixture to each of the sample tubes, mix well by pipetting (keep cold).

7. Remove bubbles and spin down. Incubate at 25°C for **1 hour** followed by 4°C for ∞ (thermal cycler with lid at 25°C or without the lid closed).

Pause Point: Samples can be stored at -80°C after this step.

Post-Ligation Purification

8. Add 54 µl Agencourt RNAClean XP beads per tube to new 1.5 ml low DNA binding tubes.

9. Add 15 µl water (DNase/RNase free) to each sample tube.

10. Mix 30 µl sample with 54 µl beads by pipetting up and down 10 times and incubate for **15 min** at room temperature for binding.

11. Place the tubes in a DynaMag™-2 Magnet and incubate at RT for **5 min** in order for the beads to separate. **Do not remove the tubes from the magnetic rack until step 16**.

12. Remove buffer by slow pipetting; make sure not to touch the beads.

13. Add 1000 µl 80% ethanol to each tube. After about 30 sec, slowly remove the ethanol by pipetting. Make sure not to touch the beads.

14. Repeat step 13 twice more, for a total of three ethanol treatments.

15. Let air dry, with the lids open, until you see the first signs of cracking. **Do not over dry the samples!**

16. Suspend beads in 10 µl water (DNase/RNase free). In this step you have to touch the beads with the pipette tip.

17. Incubate for **2 min** (not on magnetic rack).

18. Place tubes on magnetic rack for **5 min**.

19. For each sample, collect 10 µl and transfer to a 0.2 ml low DNA binding tube.

Pause Point: Samples can be stored at -80°C after this step.

11. Second cDNA Synthesis

dNTP mix (10 mM each)	SuperScript® III Reverse Transcriptase
cDNA primer (20 µM)	RNaseOUT™ Recombinat Ribonuclease Inhibitor
First Strand Buffer (5X)	Agencourt RNAClean XP
0.1 M DTT	80% ethanol

Bring the Agencourt RNAClean XP beads to RT at least 30 min before use and vortex properly before adding to the tubes.

1. Set a thermo cycler to 65°C (with heated lid at 105°C)

2. Prepare 0.2 ml low DNA binding tubes with the following reagents in each: (mix well by pipetting and keep cold)

Adapter ligated and purified aRNA	10 µl
dNTP mix (10 mM each)	1 µl
cDNA Primer (20 µM)	1 µl

3. Incubate tubes at 65°C for **5 min** and put directly on ice.

4. Add the following to a 1.5 ml tube to prepare second cDNA synthesis master mix and mix well by pipetting (keep cold):

First Strand Buffer (5X)	30 µl
0.1 M DTT	7.5 µl
Water (DNase/RNase free)	7.5 µl
SuperScript® III Reverse Transcriptase	7.5 µl
RNaseOUT™ Recombinat Ribonuclease Inhibitor	7.5 µl

5. Add 8 µl of the second cDNA synthesis master mix to each of the 0.2ml sample tubes. Mix well by pipetting and keep cold.

6. Remove bubbles and spin down. Incubate at 50°C for **1 hour** followed by 4°C for ∞ (thermal cycler with heated lid at 70°C).

Pause Point: Samples can be stored at -20°C after this step.

cDNA Purification

7. Add 54 µl Agencourt RNAClean XP beads per tube to new 1.5 ml low DNA binding tubes.

8. Add 10 µl water (DNase/RNase free) to each sample tube.

9. Mix 30 µl sample with 54 µl beads by pipetting up and down 10 times and incubate for **15 min** at RT for binding.

10. Place the tubes in a DynaMag™-2 Magnet and incubate at RT for **5 min** in order for the beads to separate. **Do not remove the tubes from the magnetic rack until step 15**.

11. Remove buffer by slow pipetting. **Do not touch the beads**.

12. Add 1000 µl 80 % ethanol to each tube. After **30 seconds**, slowly remove the ethanol by pipetting. **Do not touch the beads**.

13. Repeat step 12 twice more, for a total of three ethanol treatments.

14. Let air dry, with the lids open, until you see the first signs of cracking. **Do not over dry the samples!**

15. Suspend beads in 10 µl water (DNase/RNase free). In this step you have to touch the beads with the pipette tip.

16. Incubate for **2 min** (not on magnetic rack).

17. Place tubes on magnetic rack for **5 min**.

18. For each sample, collect 10 µl and transfer to a new 1.5 ml low DNA binding tube.

Pause Point: Samples can be stored at -20°C after this step.

12. qPCR Sample Quantification

2x KAPA mix
EvaGreen Dye, 20X in water
PCR InPE mix (InPE 1: 25 µM and InPE 2: 0.5 µM)
PCR Index 1 (25 µM)

1. Prepare qPCR reaction buffer by adding the following reagents to a tube, mix well by pipetting:

2x KAPA mix	37.5 µl
EvaGreen Dye, 20X in water	3.8 µl
Water (DNase/RNase free)	15.8 µl
PCR InPE mix (InPE 1: 25 µM and InPE 2: 0.5 µM)	1.5 µl
PCR Index 1 (25 µM)	1.5 µl

2. Add 8 µl of the qPCR reaction buffer to each of 6 wells in a qPCR plate, ensure at least one empty well between each sample to avoid cross contamination.

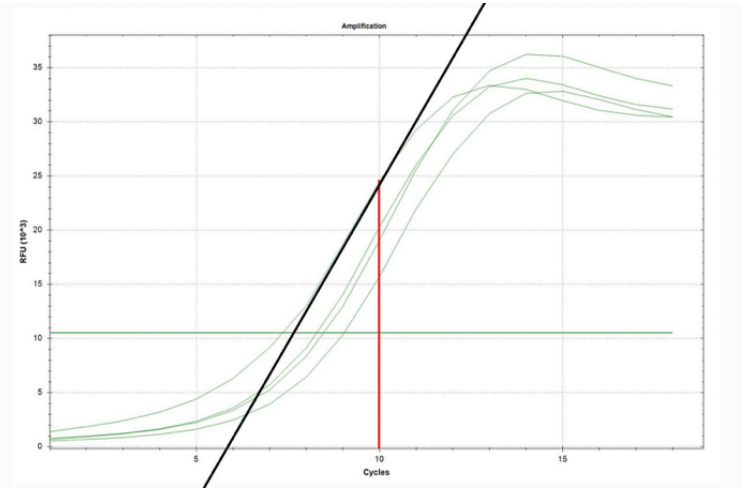
3. Add 2 µl of sample to one of the wells containing reaction buffer (10 µl total). Mix well by pipetting.

4. Seal the plate and spin down.

5. Run qPCR (with heated lid at 105°C):

98°C for 3 min
25 cycles:
98°C for 20 sec
60°C for 30 sec
72°C for 30 sec

6. Determine the number of cycles (X) for each sample required for indexing based on the maximum number of cycles you can amplify the material with, while still being in the exponential phase (usually 3-4 cycles from the maximum). In the example below, 10 cycles was chosen according to this strategy.



13. PCR Amplification

2x KAPA mix
PCR InPE mix (InPE 1: 25 µM and InPE 2: 0.5 µM)
PCR Indexes (25 µM) 1-12, choose 6: one for each sample
Agencourt AMPure XP
80% ethanol
EB Buffer (Qiagen)

1. Add the following reagents to each of six tubes, mix well by pipetting:
   (Y is one of the indexes, use a different index for each tube)

2x KAPA mix	25 µl
PCR InPE mix (InPE 1: 25 µM and InPE 2: 0.5 µM)	1 µl
PCR Index Y (25 µM)	1 µl
Water (DNase/RNase free)	13 µl

2. For each PCR mix, add 20 µl to a new 0.2 ml PCR tube.

3. Add 5 µl of sample to its respective PCR tube.

4. Remove bubbles and spin down the tubes.

5. Run PCR (thermo cycler with heated lid at 105°C):

1. 98°C for 3 min
2. X cycles: • 98°C for 20 sec • 60°C for 30 sec • 72°C for 30 sec
3. 72°C for 5 min
4. 4°C for ∞

6. Add 15µl water (RNase/DNase free) to each sample.

Pause Point: Samples can be stored at -20°C after this step.

Post-PCR Purification

7. Add 32 µl AMPure XP beads to each of six new 1.5ml low binding tubes.

8. Mix 40 µl sample with 32 µl beads by pipetting up and down 10 times and incubate for **15 min** in RT for binding.

9. Place the tubes in a DynaMag™-2 Magnet and incubate at RT for **5 min** in order for the beads to separate. **Do not remove the tubes from the magnetic rack until step 14**.

10. Remove buffer by slowly pipetting. **Do not touch the beads**.

11. Add 1000 µl 80% ethanol to each tube. After **30 seconds**, slowly remove the ethanol by pipetting. **Do not touch the beads**.

12. Repeat step 11 twice more, for a total of three ethanol treatments.

13. Let air dry, with the lids open, until you see the first signs of cracking. **Do not over dry the samples!**

14. Resuspend beads in 20µl EB Buffer. In this step you have to touch the beads with the pipette tip.

15. Incubate for **2 min** (not on magnetic rack).

16. Place tubes on magnetic rack for **5 min**.

17. For each sample, collect 20 µl and transfer to a new 1.5 ml low DNA binding tube.

Pause Point: Samples can be stored at -20°C after this step.

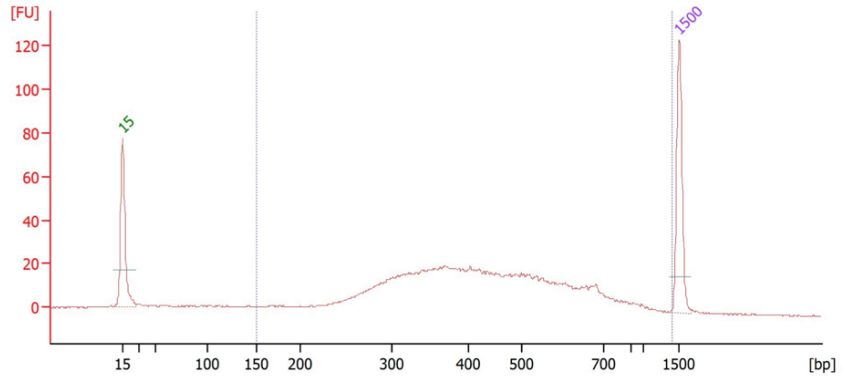
14. Library Quality Control

Qubit DNA HS kit
Agilent DNA 1000 Kit/High Sensitivity Kit
Elution Buffer

1. Measure the concentration using a Qubit (DNA HS). Use 2µl of sample for the measurement.

2. Analyze the samples using a Bioanalyzer DNA 1000 kit or Bioanalyzer DNA High Sensitivity kit and measure the average length by clicking on the “Region Table” tab in the software and setting the region to between 150 bp and 1400 bp.

3. The finished libraries should look like this:



4. For sequencing using Illumina sequencing platforms: Dilute the samples according to Illumina’s recommendations in Elution buffer (EB).

Appendix

Tissue Recommendations

The following table gives recommendations on how to treat different tissue types.

Please note that these conditions can vary between samples and laboratories, thus we strongly recommend performing a TO experiment prior to beginning LP.

In general our experience is that more fibrous tissue requires longer tissue removal steps.

Tissue Type	Mouse Brain	Human Breast Cancer	Mouse Heart	Mouse Lung
Sectioning Thicness	10 µm	16 µm	10 µm	10 µm
Hematoxylin incubation	7 minutes	7 minutes	7 minutes	7 minutes
Eosin Y incubation	60 seconds	60 seconds	60 seconds	60 seconds
Permeabilisation (Pepsin/HCl)	6 minutes	10 minutes	8 minutes	10 minutes
Tissue Removal RLT/ Beta-Mercaptoethoanol 1X (10 µl)	-	-	1 hour	1 hour
2X (20 µl)	-	-	-	-
3X (30 µl)	-	1 hour	-	-
4X (40 µl)	-	-	-	-
Tissue Removal Proteinase K	1 hour	1 hour	1 hour	1 hour

Preparing Stock Reagents and Buffers

Pre-Permeabilisation Reagent (50 U/µl collagenase)

1. Add HBSS buffer to the collagenase powder to reach a final concentration of 50 U/µl. Each collagenase powder bottle contains a bottle specific amount of U/mg.

2. Filter the collagenase solution through a 0.45 µm low protein binding filter.

3. Make 10 µl aliquots and store them at -20 °C.

Permeabilisation Reagent (0.1 % pepsin in HCl)

1. Weigh 0.1 g pepsin

2. Dilute the pepsin to 0.1% as follows:

   - Add 1 ml H2O to the tube with pepsin and mix well by pipetting.
   - Take 60 µl of the diluted pepsin solution and add to 5940 µl 0.1 M HCl.
   - Make 500 µl aliquots and store them at -20 °C.

Actinomycin D (5 µg/µl)

Work in a ventilated hood.

1. Add 400 µl of 100% filtered DMSO to the bottle containing 2 mg actinomycin D and vortex properly until all powder has dissolved.

2. Make 10 µl aliquots and store them at -20 °C.

0.1X SSC

Prepare 0.1X SSC by mixing the following:

SSC (20x) buffer	10 ml
Water (DNase/RNase free)	1990 ml

0.2X SSC

Prepare 0.2X SSC by mixing the following:

SSC (20x) buffer	20 ml
Water (DNase/RNase free)	1980 ml

2x SSC/0.1 % SDS buffer

Prepare buffer by mixing the following:

SSC (20x) buffer	200 ml
10% SDS in water	20 ml
Water (DNase/RNase free)	1780 ml

0.45 M Tris/Acetic Acid buffer

1. Weigh 55 g of Tris base powder and dissolve it in 500 ml RNase and DNase free water.

2. Adjust pH to 6.0 by carefully adding 100 % acetic acid.

3. Fill up with RNase and DNase free water to 1000 ml.

PCR InPE mix (InPE 1: 25 µM and InPE 2: 0.5 µM)

Prepare by mixing the following in a tube:

InPE 1 (50 µM)	7.5 µl
InPE 2 (1 µM)	7.5 µl

Required Consumables

The following list contains reagents necessary to perform an LP experiment. The list might not be complete, so please check the detailed LP Protocol when planning an LP experiment.

Consumable	Supplier
Optional: Lysing kit e.g. Precellys Lysing Kit Article # KT03961-1-1-003.2	Bertin Instruments
Optional: RNA Extraction kit e.g. RNeasy Plus Mini Kit Article # 74134	Qiagen
Optional: Human Reference RNA Article # 740000	Agilent Technologies
Optional: RNA fragmentation kit e.g. Magnesium RNA Frag-mentation Module Article # E6150S	New England Biolabs
Optional: RNeasy MinElute Cleanup Kit Article # 74204	Qiagen
Optional: Qubit™ RNA BR Assay Kit Article # Q10210	Thermo Fisher Scientific
Formaldehyde solution, 36.5-38% in H_2O e.g. Article #F8775-25ML	General Laboratory Supplier Sigma-Aldrich
Phosphate buffered saline (PBS)	General Laboratory Supplier
Isopropanol (2-Propanol)	General Laboratory Supplier
Mayer's Hematoxylin Solution e.g. Article # S3309	General Laboratory Supplier Dako
Bluing Buffer e.g. Article # CS702	General Laboratory Supplier Dako
Tris base (Powder)	General Laboratory Supplier
Eosin Y (Aqueous), 0.5% in water e.g. Article # HT110216-500ML	General Laboratory Supplier Sigma-Aldrich
Glycerol (85%)	General Laboratory Supplier

Ethanol 96-99%	General Laboratory Supplier
HBSS buffer Article # 14025-050	Thermo Fisher Scientific
BSA (20 mg/ml) Article # B9000S	New England Biolabs
Collagenase Article # 17018-029	Thermo Fisher Scientific
Low protein binding syringe filters e.g. Article # CLS431219-50EA	General Laboratory Supplier Sigma Aldrich
Pepsin Article # P7000-25G	Sigma Aldrich
0.1 M HCl	General Laboratory Supplier
Actinomycin D Article # A1410-2MG	Sigma Aldrich
DMSO	General Laboratory Supplier
Chemical resistant syringe filters e.g. Article # CLS43122250EA	General Laboratory Supplier Sigma Aldrich
dNTP (10 mM each) Article # R0191	Thermo Fisher Scientific
SuperScript III Reverse Transcriptase Article # 18080044	Thermo Fisher Scientific
5x First Strand Buffer (comes with SuperScript® III)	Thermo Fisher Scientific
DTT (100 mM) (comes with SuperScript® III)	Thermo Fisher Scientific
RNaseOUT™ Recombi- nat Ribonuclease Inhibitor Article # 10777019	Themo Fisher Scientific
Buffer RLT Article # 79216	Qiagen
β-mercaptoethanol	General Laboratory Supplier
Proteinase K Article # 19131	Qiagen

Buffer PKD Article # 1034963	Qiagen
SSC (20x) e.g. Article # S6639-1L	General Laboratory Supplier Sigma Aldrich
SDS e.g. SDS 10% Article # 71736-100ML	General Laboratory Supplier Sigma Aldrich
Second Strand Buffer (5X) Article # 10812014	Thermo Fisher Scientific
USER™ Enzyme Article # M5505S	New England Biolabs
DNA Polymerase I Article # 18010025	Thermo Fisher Scientific
Ribonuclease H Article # 18021071	Thermo Fisher Scientific
T4 DNA Polymerase Article # M0203S	New England Biolabs
EDTA 500 mM	General Laboratory Supplier
MEGAscript® T7 Transcription Kit Article # AM1333	Thermo Fisher Scientific
SUPERase In™ RNase Inhibitor Article # AM2694	Thermo Fisher Scientific
Agencourt RNAClean XP Article # A63987	Beckman Coulter
Agencourt AMPure XP Article # A63880	Beckman Coulter
T4 RNA Ligase 2, truncated Article # M0242L	New England Biolabs
10X T4 RNA Ligase Buffer (comes with Ligase)	New England Biolabs
RNase inhibitor, murine Article # M0314S	New England Biolabs
2x KAPA mix Article # KK2601	Techtum

EvaGreen Dye, 20X in water, Article # 31000	Biotium
Buffer EB Article # 19086	Qiagen
Agilent DNA 1000 Kit Article # 5067-1504	Agilent Technologies
Agilent High Sensitivity DNA Kit Article # 5067-4626	Agilent Technologies
Agilent RNA 6000 Pico Kit Article # 5067-1513	Agilent Technologies
Qubit dsDNA HS Assay Kit Article # Q32851	Thermo Fisher Scientific
Qubit assay tubes Article # Q32856	Thermo Fisher Scientific
SlowFade® Gold Antifade Mountant Article # S36936	Thermo Fisher Scientific

Required Oligos

Name	**Sequence** (according to IDT nomenclature)
aRNA Ligation Adapter	/5rApp/AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC/3d-dC/
cDNA Primer	GTGACTGGAGTTCAGACGTGTGCTCTTCCGA
PCR InPE 1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTA-CACGACGCTCTTCCGATCT
PCR InPE 2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
PCR Index 1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTC
PCR Index 2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTC
PCR Index 3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTC
PCR Index 4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTC
PCR Index 5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTC
PCR Index 6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTC
PCR Index 7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTC
PCR Index 8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTC
PCR Index 9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTC
PCR Index 10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTC
PCR Index 11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTC
PCR Index 12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTC
Cyanine-3 A Probe	/5Cy3/AGATCGGAAGAGCGTCGTGT
Cyanine-3 Frame Probe	/5Cy3/GGTACAGAAGCGCGATAGCAG

Required Equipment

The following table lists equipment required to perform an LP experiment:

Equipment	**Supplier**
Hybridization Cassette* ("**Slide Module**") ProChamber 16-well # RD482874 (as seen on page 7.)	Grace Bio-Labs (contact Grace Bio-Labs sales support to order)
Hybridization Cassette* ("**Slide Module**") ProPlate Multi-Well Chamber # RD482441 (disposable, lower cost alternative to ProChamber)	Grace Bio-Labs (contact Grace Bio-Labs sales support to order)
Optional: Tissue Homogenizer e.g. Minilys Homogenizer Article # P000673-MLYSO-A	General Laboratory Supplier Bertin Instruments
Bright-field microscope	
Fluorescent microscope	
Vortex	General Laboratory Supplier
Microcentrifuge for 1.5 mL tubes	General Laboratory Supplier

Thermostat for 1.5 ml tubes	General Laboratory Supplier
Thermomixer for plates e.g. Thermomixer® C + smartblock for pl ates Article # 5382000015 + # 5363000039	General Laboratory Supplier Eppendorf
Optional: Microcentrifuge for glass slides with the dimensions 25 mm x 75 mm x 1 mm	General Laboratory Supplier
2100 Bioanalyzer Desktop Bundle Article # G2940CA	Agilent Technologies
qPCR System e.g. CFX Connect Article # 1855201	Biorad
Thermal cycler with heated lid for PCR	
DynaMag™-2 Magnet Article # 12321D	Thermo Fisher Scientific
Axygen Single Well High Profile Reagent Reservoir Article# 14-222-424	Fisher Scientific

*we list two alternative hybridization cassettes to choose from. You **do not** need to order both. The ProChamber and ProPlate slide modules are compatible with both LP and TO slides, but the ProPlate is single-use only.