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Spatial Transcriptomics Protocol V.2

Michael Eadon[1], Ricardo Melo Ferreira[1], Ying-Hua Cheng[1], Tarek Ashkar[1]

[1]Indiana University School of Medicine

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KPMP
Tech. support email: **info@kpmp.org**

Michael Eadon

The tissue in OCT undergoes cryosectioning, affixment to the cDNA capture slide, H+E staining, 20x Keyence imaging, tissue permeabilization, RNA capture, and cDNA synthesis. Data is analyzed in Loupe browser and in R prior to uploading to the KPMP Data Lake.

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Stephanie Grewenow

protocol ,

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Nov 16, 2021 Stephanie Grewenow University of Washington

Nov 24, 2021 Michael Eadon

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Protocol Quality Control Metrics

A number of our QC metrics have been recommended by 10X Genomics. We will adhere to the recommended commercial standards, specifically an input RIN of greater than 7. Based on our preliminary data, it may be possible to interrogate samples of reduced input quality and still obtain adequate sample output quality for analysis. If bulk RIN is higher than 3.8 we intend to procced with interrogation, flagging the sample if it meets output QC metrics established by 10X genomics. The QC metrics include:

- Bulk RNA quality, Ideal RIN should be greater than 7, according to 10X genomics. Samples with a RIN between 3.8 and 6.9 will be interrogated and flagged if they meet downstream QC metrics. Samples with a Bulk RIN less than 3.8 will not be interrogated (No-Go criteria). We chose 3.8 because two samples with a RIN of 3.8 met the 10x genomics defined QC output metrics. There is some trade-off in using samples with lower input QC metrics (i.e. a RIN between 3.8 and 6.9), but most samples meet the output QC metrics defined by 10x.
- Tissue affixment without folds or overlap – if folded, wipe and re-apply
- Keyence image visual inspection – if inadequate resolution, reimage single specimen. The visual inspection will be performed by two individuals including the core microscopist and KPMP biologist. Minimum standards include the ability to identify the following four features: sharp nuclei, glomeruli, proximal tubules, and distal tubules.
- Post-permeabilization RNA quality and quantity: sigmoid curve shape on RT-PCR with sufficient RNA before cycle 20.
- cDNA quality – Assess fragmentation. cDNA size should be approximately 500 bp of higher. This is not a hard Go, No-Go criteria.
- Sequencing Q30 – better than 90%.
- Transcript mapping – majority of reads (more than 50%) must map to spots with tissue overlying. Sample will be flagged if not meeting this threshold.
- Reads mapped confidently to Exons should be above 30%. Sample will be flagged if not meeting this threshold.
- Picture of alignment process is taken to ensure fiducials correspond – correct informatically if misaligned. Fiducials are defined as spots around the periphery of the capture area to allow image alignment.
- Expected differential expression of marker genes in spot clusters.
- Sequencing saturation will be tracked to gauge the appropriate depth of sequencing and assess for drift. However, this is not a quantitative output metric.

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RNAse Away (Ambion, Cat #10328011)
Leica PPS-membrane slides (Leica, Cat# 11600294)
small slide box (Simport, M950-4MA)
50mL RNAse free conical centrifuge tubes (Cat#91050)
Visium library preparation slides (1000185)
Methanol (Millpore Sigma, 34860)
isopropanol (Millpore Sigma, I9516-25ml)
Hematoxylin, Mayer's (Agilent, S330930-2)
RNase free water (Thermo Fisher, AM9937)
Bluing Buffer (Dako, CS702)
Eosin Mix (Sigma, HT110216-500ml)

Cryosectioning Procedure 30m

1 Specimens should be handled wearing clean gloves treated with RNAse Away or with a clean RNAse Away treated forceps.

Perform all work in clean manner with clean disposable gloves and face mask. Ensuring the cleanliness of all surfaces (including RNAse Away, Ambion, Cat #10328011), this is particularly critical when multiple technologies are cryosectioned in the same session and a portion of slides cut will be for RNA technologies. This protocol is to be used with kidney tissue preserved in OCT and stored in **-80 °C** .

2 Cool Cryostat to **-22 °C** . Clean the work surfaces with RNAse away and install a new cutting blade.

3 Place a clean small slide box inside the cryostat chamber to store slides containing freshly cut tissue.

4 Clean and treat the tissue holder will RNAse away and put it in the cryostat chamber . Adhere the OCT specimen to a tissue holder and allow it to equilibrate for a few minutes to reach the chamber temperature and strengthen the adhesion between the OCT block and the holder. This process can be aided via use of a heat extractor. The tissue holder was cleaned and treated with RNAse Away and cooled down.

5 In parallel, allow the cDNA capture slide (Visium product code #1000184) to equilibrate at **-20 °C** .

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6 Place the specimen in the cryostat with the tissue holder and cut at **10 µm** thickness (1 section).

> The section may be scored with a razor blade (shallow <1 mm deep) if the biopsy length exceeds 6.5 mm in order to allow the whole biopsy to be included in the capture area.

7 Try to avoid overlap with fiducials surrounding the capture area. If that is not possible, this can be adjusted informatically after sequencing if there is overlap. A minimum of 2 corners must be clear of tissue.

8 Store completed slides at **-80 °C** in sealed 50mL RNAse free conical centrifuge tubes (Cat#91050). Use slides within 48 hours of cryosectioning.

9 Process specimens in batches of four because there are 4 capture areas per slide.

10 If tissue is folded or misplaced, the slide can be reset using Visium Spatial Slide Reset Protocol (CG000332 • Rev B) and reuse.

Fixation and Hematoxylin and Eosin staining 36m

11 Use the Visium library preparation slide with affixed OCT sections and perform H+E staining.

12 Chill **40 mL** Methanol in a 50-ml centrifuge tube to **-20 °C** .

13 Place a Thermocycler Adaptor on thermal cycler set at **37 °C** and equilibrate for **00:05:00** . Heating the thermal cycler lid is not required. 5m

14 Place slide on the Thermocyler Adaptor with the active surface facing up and incubate **00:01:00** at **37 °C** . DO NOT close the thermal cycler lid, as the thermal cycler lid is 1m

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normally heated to a higher temperature and it may touch the tissue sections when closed.

15 Completely immerse the slide in the prechilled methanol. Secure the tube cap. Incubate upright for **00:30:00** at **-20 °C** . 30m

16 Remove slide from methanol and wipe excess liquid from the back of the slide without touching the tissue sections.

17 Add **500 µL** isopropanol to uniformly cover all tissue sections on the slide. Incubate **00:01:00** at **Room temperature** . 1m

18 Drain reagent and air dry the slide for **00:06:00** . 6m

19 Add **1 mL** Hematoxylin to uniformly cover all tissue sections on the slide. Incubate **00:07:00** at **Room temperature** . 7m

20 Discard reagent and rinse slide sequentially in RNase free water. Wipe away excess liquid.

21 Add **1 mL** Bluing Buffer to uniformly cover all tissue sections. Incubate **00:02:00** at **Room temperature** . 2m

22 Discard reagent and rinse slide sequentially in RNase free water. Wipe away excess liquid.

23 Add **1 mL** Eosin Mix to uniformly cover all tissue sections. Incubate **00:01:00** at **Room temperature** . 1m

24 Discard reagent and rinse slide sequentially in RNase free water. Wipe away excess liquid.

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11m

25 Allow slide to air dry for **00:06:00** then incubate the slide on the Thermocycler Adaptor with the thermal cycler lid open for **00:05:00** at **37 °C** prior to imaging

Imaging 24m

26 After the specimen is fixed and stained with H+E reagents it is then imaged on a Keyence BZ-X810 scanning microscope at 10x or 20x resolution in the IU O'Brien Center for Microscopy.

> The slide harbors an array containing capture probes that bind to RNA with barcodes enabling the mapping of mRNA expression back to 55 µm diameter "spots" on the section.

27 Brightfield images of stained sections in the fiducial frames are collected as mosaics of 20x fields using a Keyence BZ-X810 microscope equipped with a Nikon 10X CFI Plan Fluor objective.

24m

28 Including set-up time, **00:24:00** was required to image 4 sections. Image quality is assessed by visual inspection prior to RNA isolation and a given section can be re-imaged if necessary (~6 min needed).

29 Mosaics are stitched.

cDNA capture and sequencing 12m

30 Bulk RNA quality is assessed on an adjacent section with an Agilent bioanalyzer prior to sectioning the tissue for spatial transcriptomics. Ideal RIN should be above 7, although some specimens may be able to yield adequate data with a RIN as low as 3.8.

12m

31 Permeabilize stained tissue sections for **00:12:00** , mRNA was released to bind oligonucleotides on the capture areas with a test for RNA quality/quantity after permeabilization and isolation using real time PCR amplification from the Visium library prep slide.

32 cDNA synthesis - reverse transcription, second strand synthesis, denaturation, cDNA

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amplification, SPRIselect cDNA cleanup. cDNA quality is assessed by Agilent bioanalyzer.

33 Prepare and sequence the cDNA libraries on an Illumina NovaSeq 6000 with 28bp+120bp paired-end sequencing mode.

34 Using a Loupe Browser, map expression data to each “spot” and visualize overlying the histologic image

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