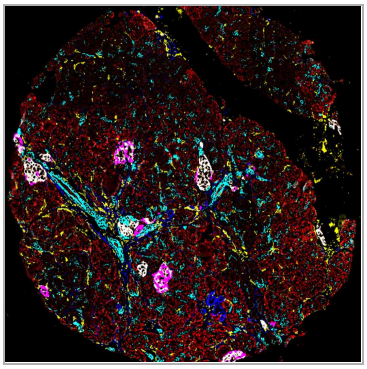

MAR 12, 2023



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We use this protocol and it's working

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# CODEX ® Multiplexed Imaging – tissue staining in TMA and whole tissue FFPE sections

Anna Martinez Casals[1,2], Inna Sitnik[1,2], Christian M. Schürch[3], Mikael Malmqvist[4], Patrick E Macdonald[5], Emma Lundberg[1,2]

[1]Science for life laboratory; [2]KTH - Royal Institute of Technology; [3]Stanford University; [4]Atlas Antibodies; [5]University of Alberta

Anna Martinez Casals
Science for life laboratory ; KTH - Royal Institute of Techn...

## ABSTRACT

CODEX technology allows for highly multiplexed analysis of + 40 proteins on the same section relying on DNA-conjugated antibodies being commercialized by Akoya Biosciences (former name CODEX®, current name PhenoCycler™). This detailed protocol describes an adapted protocol, from CODEX User Manual Rev C , for tissue staining in Tissue MicroArrays (TMA) and whole tissue Formalin Fixed Paraffin Embedded (FFPE) sections to run into the CODEX system which is used in Emma Lundberg research group at Science for Life Laboratory; KTH - Royal Institute of Technology.

## IMAGE ATTRIBUTION

Tissue core from a TMA FFPE pancreas section generated in the ESPACE project, Human Cell Atlas (HCA) initiative.

## MATERIALS

Product suggestion: Cover glasses square, Marienfeld, cat# 102052.
Product suggestion: Wipes, Kimtech™ Science - Precision, cat# 115-2221.
Product suggestion: VWR® W10 , Slide warmer/dryer, VWR, cat# 720-2422.
Product suggestion: Coverglass Staining Rack, Electron Microscopy Sciences cat# 72240. It is a coverslip staining rack made from polished stainless steel. Specially designed to be used with coplin jars holding five of 22 x 24 mm coverglass (suitable for the 22 x 22 mm).
Product suggestion: EasyDip™ slide staining kit, Simport Scientific, cat# M906-12AS.
Product suggestion: TintoRetriever - heat retrieval system, Bio SB.
Product suggestion: 2 items of A4 Ultra bright LED light box pad 25.000 lux.
Product suggestion: Cover Slip Forceps, F.S.T, cat# 11251-33.
Product suggestion: StainTray slide staining system, Sigma, cat# Z670146-1EA.
Product suggestion: Corning® Ice Bucket round, VWR (Corning), cat# 75779-976.

## SAFETY WARNINGS

Refer to the SDS of each of the solvents and chemicals used in this protocol for safe lab practices. Consult your organization to learn the appropriate way to dispose the chemical waste.

## ETHICS STATEMENT

Review the ethical permits needed for the project and ensure to have all the documentation in place before starting any experiment.

The pancreas tissue core used, to generate the thumbnail image of this protocol, was provided in the framework of ESPACE project - Human Cell Atlas (HCA) initiative. An ethical permit linked to this study was applied and approved by the Swedish Ethics Review authority - *Etikprövningsmyndighetens* (Dnr 2020-02507).

## BEFORE START INSTRUCTIONS

Review the protocol before starting it to ensure having all the material needed.

# Tissue preparation: I. Coverslip coating & sectioning

1h 5m

**1** 1d

Place coverslips in a glass beaker and cover them with Poly-l-lysine, 0.1% (wt/vol) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P8920** . Ensure all the coverslips are fully immersed in the solution avoiding overlapping, add a plastic wrap to prevent evaporation and let them coat Overnight .

Note

**Product suggestion**: Cover glasses square, Marienfeld, cat# 102052.

**2** 1m

Carefully remove the poly-l-lysine, cover the coverslips with Milli-Q water, stir slowly and let them settle during 00:00:30 for a first wash. After the first wash, slowly remove the Milli-Q water.

**3** 10m

Repeat the go to step #2 for a total of 6 washes .

**4**

Batch by batch, start transferring coverslips from the glass beaker to a sterile Petri dish with Milli-Q water. Place 2 tissue wipes on the bench: take each individual coverslip and place it in a tissue

wipe, once it is dried from one side move it to the next tissue wipe to dry the other side. Repeat the process with all the coverslips, changing the tissue wipes once they are wet.

Note

Try to avoid using tissues that may leave traces of cellulose. **Product suggestion**: Wipes, Kimtech™ Science - Precision, cat# 115-2221.

5 Place the completely dried coverslips in a new Petri plate to store them at Room temperature till 2 months (label the Petri dish with the date). It is recommended that coverslips are coated with poly-L-lysine at least 2 days prior to tissue sectioning. 5m

6 Section the TMA or whole tissue block on the coated coverslips, trying to center it as much as possible. Recommended 5-10 µm /section.

## Tissue preparation: II. Tissue treatment

7 Place the coverslip (tissue facing up, see Fig. 1) in a slide warmer and bake it at 55 °C during 01:00:00 1h

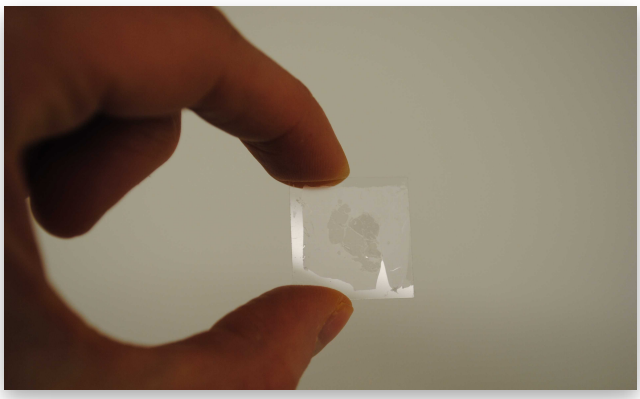


Fig. 1 | Coverslip with FFPE tissue section, easy to recognize the tissue side due to the presence of paraffin.

Note

**Product suggestion**: VWR® W10 , Slide warmer/dryer, VWR, cat# 720-2422.

8 Transfer the coverslip to a coverslip staining rack and let it cool down for 00:05:00 . 5m

Note

**Product suggestion**: Coverglass Staining Rack, Electron Microscopy Sciences cat# 72240. It is a coverslip staining rack made from polished stainless steel. Specially designed to be used with Coplin jars holding five of 22 x 24 mm coverglass (suitable for the 22 x 22 mm).

9 Start the deparaffinization and hydration steps (Fig. 2): place the coverslip staining rack carefully in each of the next solvents, following the order, during 00:05:00 . Ensure to close the lid of each of the containers to avoid evaporation.

Note

**Product suggestion**: EasyDip™ slide staining kit, Simport Scientific, cat# M906-12AS.


Fig. 2 | Slide staining station includes one anodized aluminum rack along with six assorted color jars (two white ones) and one slide staining rack. The aluminum holder can hold up to 6 staining jars. The anodized surface is resistant to rust, corrosion, and abrasion.

9.1 HistoChoice Clearing agent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #H2779 1L** 5m

9.2 HistoChoice Clearing agent **Merck MilliporeSigma (Sigma-Aldrich) Catalog #H2779 1L** 5m

9.3 Ethanol absolute ≥99.8% AnalaR NORMAPUR® ACS Reag. Ph. Eur. analytical reagent **VWR International Catalog #20821.330P** 5m

9.4 Ethanol absolute ≥99.8% AnalaR NORMAPUR® ACS Reag. Ph. Eur. analytical reagent **VWR International Catalog #20821.330P** 5m

9.5 90% Ethanol prepared with ddH2O. 5m

9.6 Meanwhile prepare the pressure cooker, to perform antigen retrieval step, filling it with ddH2O using the following settings: 106-110 °C and low pressure allowing to heat up. 5m

> Note
>
> **Product suggestion**: TintoRetriever - heat retrieval system, Bio SB.

9.7 70% Ethanol prepared with ddH2O. 5m

9.8 50% Ethanol prepared with ddH2O. 5m

**9.9** 30% Ethanol prepared with ddH2O. 5m

**9.10** ddH2O. 5m

**9.11** ddH2O. 5m

**9.12** Prepare the 1x citrate buffer solution in the container from the heat retrieval system: 25 ml Citrate Buffer pH 6.0 10× Antigen Retriever **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C9999-1000ML** + 225 ml ddH2O. 5m

**9.13** Transfer the coverslip staining rack to the container with the 1x citrate buffer and place the lid on top. 1m

**9.14** Place the covered container into the heat retrieval system and set up the following settings: 114-121 °C, high pressure for 00:20:00. 20m

**9.15** Remove the container from the heat retrieval system and allow to equilibrate to RT for at least 30min (otherwise tissue detachment may occur on the slide). 30m

**9.16** Transfer the coverslip staining rack into a container with ddH2O and leave it for 00:02:00 then transfer it to a second container with ddH2O for additional 00:02:00. 4m

## Optional: Photobleaching treatment

10

Note

**Original source**: Du et al. 2019. Nature Protocols 14: 2900-2930.

**Protocol modified, to be adapted to the CODEX workflow, by**: Derek Oldridge, M.D. Ph.D. and Jonathan Belman M.D. Ph.D.

Use two LED-lights to apply directly to the tissue to reduce the tissue autofluorescence. To avoid direct exposure to the lights, use a container (Fig. 3) and place inside the LED-lights (Fig. 4) creating a sandwich where the sample will be located between them in a falcon tube.


Fig. 3 | Yellow plastic box suitable to store the LED-lights.

Note

**Product suggestion**: 2 items of A4 Ultra bright LED light box pad 25.000 lux.

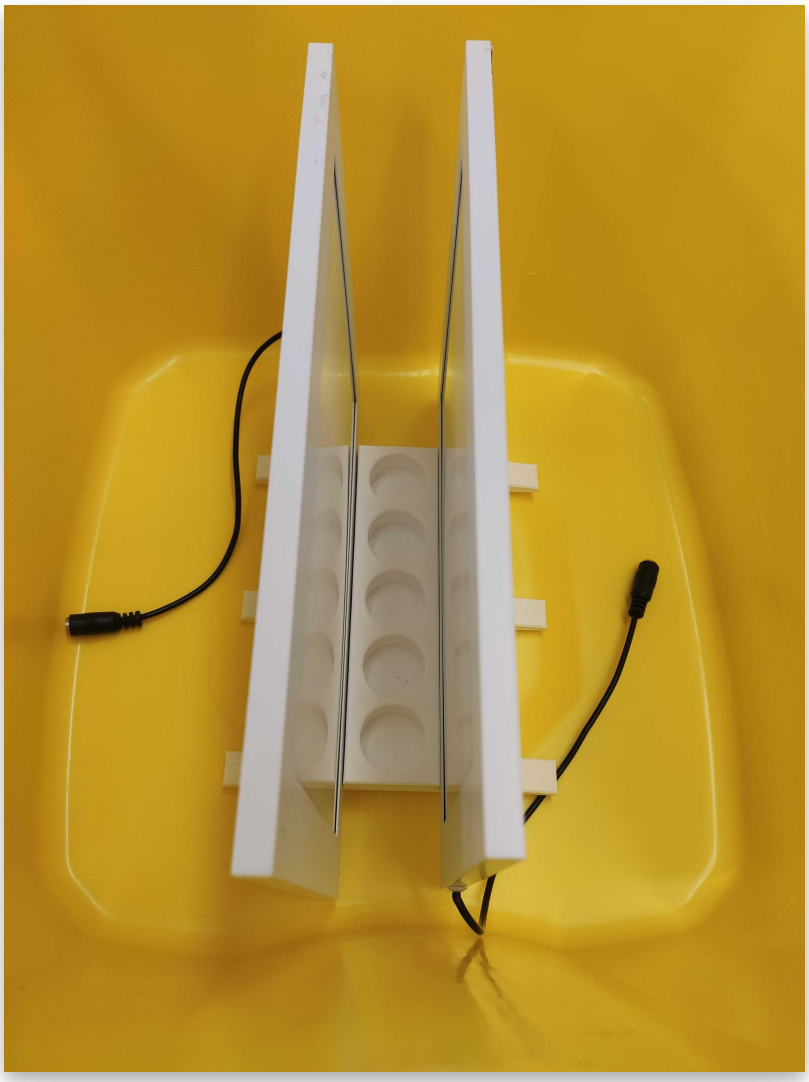


Fig. 4 | The photobleaching treatment may be performed inside the yellow plastic box to avoid direct light exposure.

**11** Prepare the photobleaching solution in a 50 ml tube: 25 ml 1x PBS + 4.5 ml 30% (w/w) Hydrogen Peroxide Solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #31642-500ML** and 0.8 ml 1M NaOH. 5m

**12** Transfer the coverslip staining rack with the sample into the 50 ml tube containing the solution and place the tube in the rack between the LED-lights. Turn them on at maximum capacity during 00:45:00 . 45m

**13** Depending of the type of tissue, the sample may undergo a second photobleaching incubation repeating go to step #11 and go to step #12 . 50m

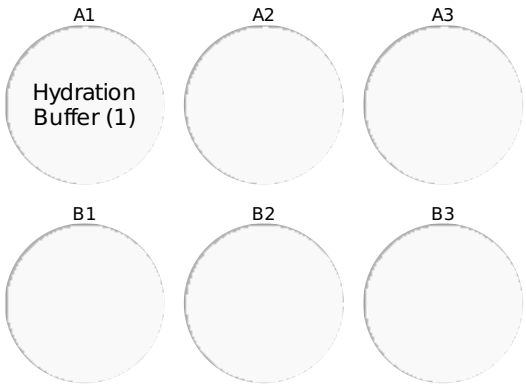
14 Wash the sample with 1x PBS during 00:03:00 . 3m

15 Repeat the go to step #14 for a total of 4 washes. 10m

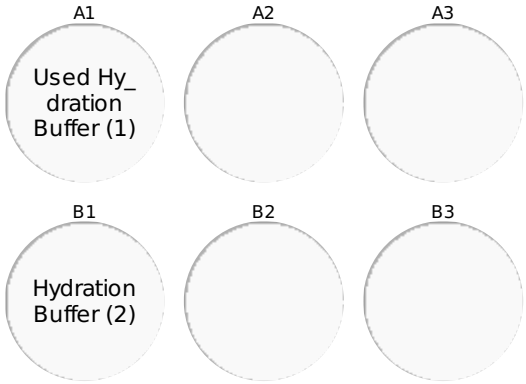
## Primary antibody staining steps

16 Using a coverslip forceps, transfer the coverslip into a well (6-well plate) with Hydration Buffer. Immerse the coverslip in the Hydration Buffer 2-3times. 3m

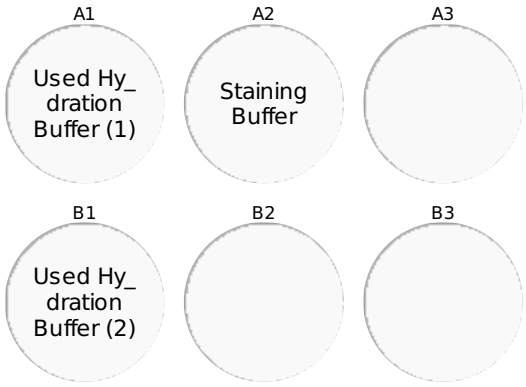
> Note
>
> **Product suggestion**: Cover Slip Forceps, F.S.T, cat# 11251-33.




17 Let the coverslip sit for 00:00:05 and transfer the coverslip to a second well with Hydration Buffer, let it sit for 00:00:05 . 15s




**18** Transfer the coverslip to the well containing the Staining Buffer and allow the sample to equilibrate for 20-30min (do not exceed the 30min). 30m




**19** Prepare the CODEX Blocking Buffer using the following components and volumes: 20m

| | CODEX Blocking Buffer | Volume (µL) for 1 coverslip |
|---|---|---|
| | Staining buffer | 45.25 |
| | N Blocker | 1.2 |
| | G Blocker | 1.2 |
| | J Blocker | 1.2 |
| | S Blocker | 1.2 |
| | Total = | 50.05 |
| | | |

Table 1 | CODEX Blocking Buffer preparation.

**20** Prepare the Antibody Cocktail solution diluting the pre-conjugated 1ary antibodies in CODEX

Blocking Buffer (final volume = 50µl). Pipette gently.

Note

**Attention**! The volume of CODEX Blocking Buffer should always be greater than 60% of the total Antibody Cocktail solution. Otherwise, sufficient blocking may not occur. If the CODEX Blocking Buffer must be less than 60% of the total Antibody Cocktail, to accommodate more antibodies, adjust the volume of the Staining Buffer down. Do not adjust the volumes of blocking components.

**21** Create an humidity chamber: fill the stainTray slide staining system (Fig. 5) with ddH2O to create a humidity environment to incubate the tissue with the 1ary antibodies. 2m

Note

**Product suggestion**: StainTray slide staining system, Sigma, cat# Z670146-1EA.

Fig. 5 | Humidity chamber with black lid for tissue incubation.

**22** Cut a rectangular piece of parafilm, roughly the size and shape of the non-label portion of a microscope slide. 2m

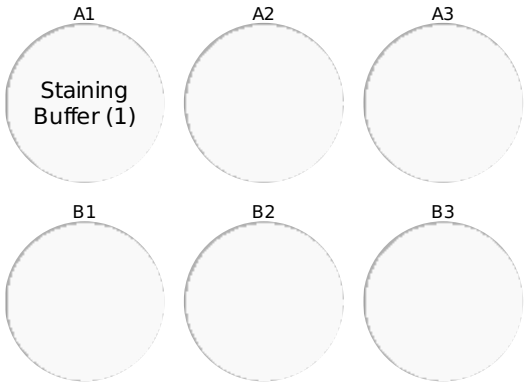
**23** Take a new microscope slide and place it in the stainTray slide staining system. Add the parafilm on top of the slide. 2m

**24** Add the total volume of Antibody Cocktail solution (50µl) in a drop on top of the parafilm and add the coverslip face down (the tissue is in contact with the solution). Close the humidity chamber. 2m

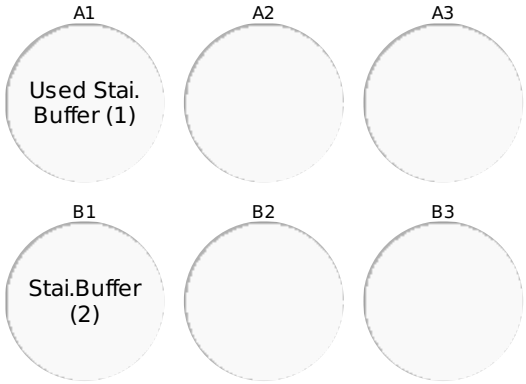
**25** Incubate at 4 °C Overnight. 1d

## Post staining steps 39m

**26** The day after: remove the coverslip from the humidity chamber, place the coverslip in a well with Staining Buffer (6-well plate) and immerse the coverslip 2-3 times. Incubate for 00:02:00. 2m




**27** Transfer the sample to a second well with Staining Buffer and incubate the samples for additional 00:02:00. 2m



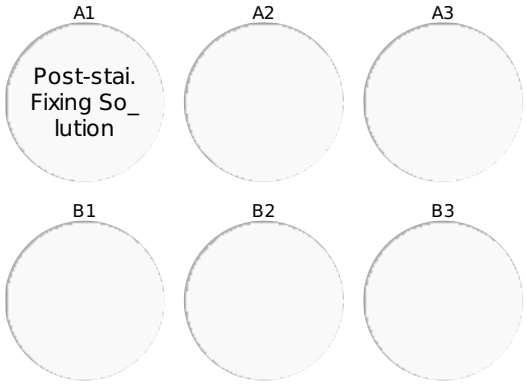

**28** Prepare the Post-Staining Fixing Solution: 500 µl 5m

Paraformaldehyde 16% (w/v) in aqueous solution methanol-free **VWR International Catalo #43368.9M**

+ 4500 µl Storage Buffer and add 5 ml of the solution in a well (new 6-well plate).

**29** Transfer the sample to the well containing the Post-Staining Fixing Solution and incubate at 10m

Room temperature for 00:10:00 .




**30** Meanwhile, add 5mL of cold methanol

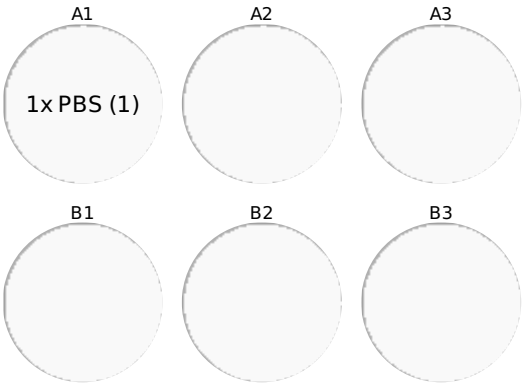
Methanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #322415** into a well (new 6-well plate) and place the plate on an On ice bucket (Fig. 6).


Fig. 6 | Setup for methanol incubation step on an ice bucket.

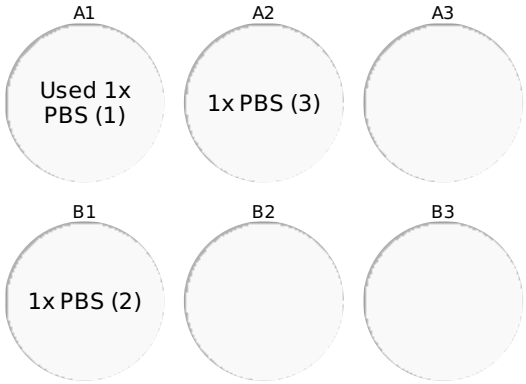
Note

**Product suggestion**: Corning® Ice Bucket round, VWR (Corning), cat# 75779-976.

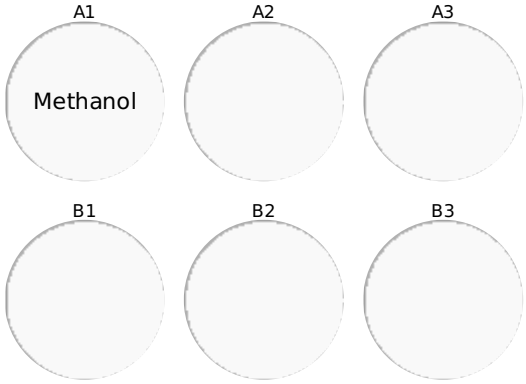
**31** Remove the coverslip from the Post-Staining Fixing Solution and place it in a well containing 1x PBS. Immerse it 2-3 times. 1m



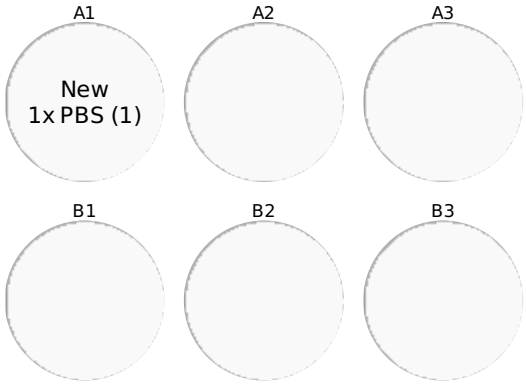

**32** Repeat go to step #31 for a total of 3 washes. 1m



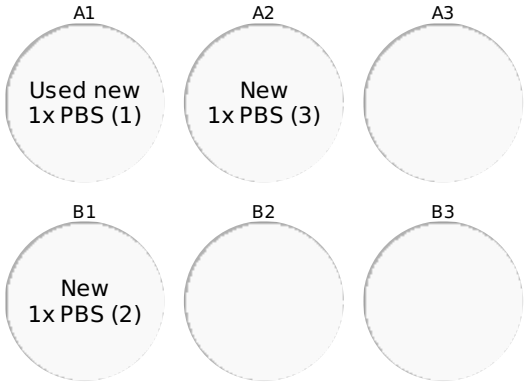

**33** Remove the coverslip from the last 1x PBS and transfer it into the ice-cold methanol well. Incubate on On ice for 00:05:00. 5m




**34** After the incubation, immediately transfer the coverslip to a well with fresh 1x PBS. Immerse the sample 2-3 times. 1m




**35** Repeat go to step #34 for a total of 3 washes. 1m



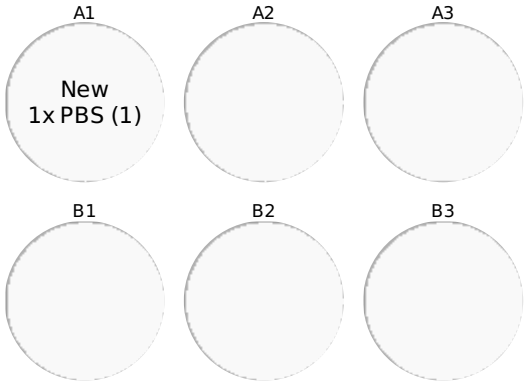

**36** Rinse and dry the StainTray slide staining system. 3m

**37** Prepare Final Fixative Solution: 1 ml 1x PBS + 20 µl of CODEX Fixative Reagent tube (do not re-freeze again) and vortex it. 3m

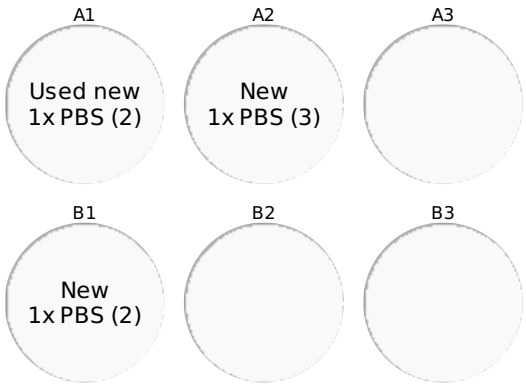
**38** Take a new microscope slide and place it in the stainTray slide staining system. Add a parafilm piece on top of the slide. 3m

**39** Pipette 200 µl of Final Fixative Solution on top of the parafilm and add the coverslip face down (the tissue is in contact with the fixative). Incubate for 00:20:00. 22m

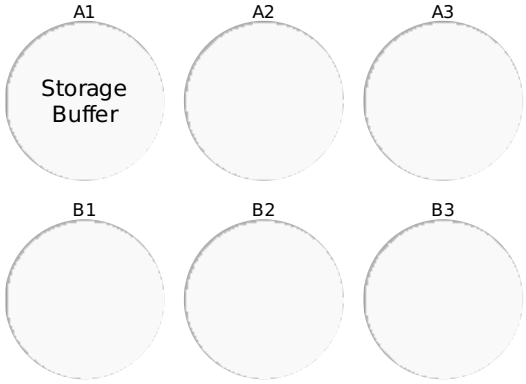
**40** Remove the coverslip from the stainTray slide staining system and transfer it to a first well of 1x PBS. Immerse the sample 2-3 times. 1m



**41** Repeat go to step #40 for a total of 3 washes. 1m




**42** Label a new 6-well plate and add 5 ml of Storage Buffer into a well: transfer the coverslip into the Storage Buffer with the tissue facing up. 5m




**43** Seal the 6-well plate and store it at 4 °C. 2m

Note

**Note**: For best results, store at 4°C for no longer than 5 days.