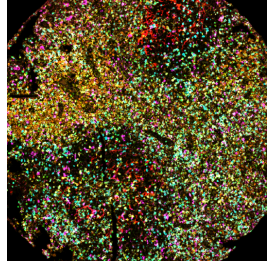




Sep 04, 2019

🌐 Automated Multi-plex Immunofluorescence with TSA for CD4, CD8, FOXP3, CD21, PD1 and CD68 in Follicular Lymphoma



DOI

dx.doi.org/10.17504/protocols.io.49ygz7w

Anna Maria Tsakiroglou¹, Catharine West¹, Sue Astley¹, Kim Linton^{1,2}, Martin Fergie¹, Richard Byers^{1,3}

¹University of Manchester; ²The Christie NHS Foundation Trust; ³Manchester Royal Infirmary Hospital



Anna Maria Tsakiroglou

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Protocol Citation: Anna Maria Tsakiroglou, Catharine West, Sue Astley, Kim Linton, Martin Fergie, Richard Byers 2019. Automated Multi-plex Immunofluorescence with TSA for CD4, CD8, FOXP3, CD21, PD1 and CD68 in Follicular Lymphoma. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.49ygz7w>

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Protocol status: Working

The protocol has been validated for agreement with single-plex assays.

Created: July 08, 2019

Last Modified: September 04, 2019

Protocol Integer ID: 25624

Keywords: Multiplex, Immunofluorescence, Immunoassay, Follicular lymphoma, Lymphocytes, multiplex immunofluorescence, multiplex immunofluorescence, protocol for multiplex immunofluorescence, cd68 in follicular lymphoma observing, immune cell spatial organisation in follicular lymphoma, follicular lymphoma observing, plex immunohistochemical staining, follicular lymphoma cohort, multiplex assay, follicular lymphoma, follicular helper cell, novel biomarkers for prognosis, immune cell, novel biomarker, follicular dendritic cell, area quantification fluorescence module, tumour microenvironment, spatial organisation in the tumour microenvironment, cd21 marker, concurrent visualisation on the same tissue section, immune cell infiltration, follicle area, macrophage

Abstract

Observing immune cell infiltration and spatial organisation in the tumour microenvironment of follicular lymphoma is important for the development of novel biomarkers for prognosis and treatment selection. However the identification of multiple immune cell sub-populations is limited in routine single-plex immunohistochemical staining.

Our goal was to develop and validate an automated protocol for multiplex immunofluorescence using tyramide signal amplification, the Opal™ 7 color Kit (Akoya Biosciences) and the Ultra Discovery (Roche) autostainer, which would enable concurrent visualisation on the same tissue sections of:

- T follicular helper cells (CD4⁺)
- cytotoxic T cells (CD8⁺)
- Tregs (CD4⁺FOXP3⁺)
- Macrophages (CD68⁺)
- PD1⁺ lymphocytes
- DAPI nuclear counterstain

We aimed in addition to observe the spatial arrangement of these cells in reference to the follicle areas, therefore the CD21 marker, which is expressed in mature B cells and follicular dendritic cells located in the follicles was added in the panel.

We validated quantitatively the agreement between single-plex and multiplex assays by comparing stained area in sequential sections of a follicular lymphoma FFPE tissue micro-array (TMA). The TMA was constructed from a follicular lymphoma cohort of 44 patients collected retrospectively from the archives of the Christie NHS Foundation Trust (Manchester), with ethical permission granted by the Central Manchester Multi-centre Research Ethical Committee (03/08/016). The protocol was automated with the DISCOVERY ULTRA IHC/ISH Roche autostainer and stained areas were quantified using the Indica Labs' Area Quantification Fluorescence module (HALO software).

Statistically significant linear correlations were seen between single-plex and multiplex assays ($R^2 > 0.72$, $p < 7.1 \times 10^{-14}$, see attached) for all markers. Therefore, this multiplex immunofluorescence (mIF) protocol can be used to conserve tissue and observe immune cell spatial organisation in follicular lymphoma.

Attachments



Protocol validation....

1.4MB



Guidelines

This protocol has been validated for use with follicular lymphoma FFPE tissue sections from lymph node tumour biopsies (4µm thickness).

A new spectral library and separate validation should be carried out before use with other types of tissue where the levels of expression may vary. Instructions for detailed optimisation steps required to set up a new protocol are provided by Akoya Biosciences.

Acknowledgements:

We thank Garry Asthon, Isabel Peset-Martin and all the staff in Histology and Imaging Facilities, Cancer Research UK, Manchester, for their advice and support, as well as Ros Lloyd and Virginie Goubert in Akoya Biosciences for their helpful input. Additionally we would like to acknowledge the work of Syed Islam who kindly shared a mIF protocol using FOXP3, PD1, PDL1, CD8 and CD3, as well as Derrick Morgan who shared an optimised chromogenic protocol for the CD4 antibody; these were used as base for development of the current setup.

Materials

MATERIALS

- ⊗ Prolong Gold **Thermo Fisher Scientific Catalog #P36930**
- ⊗ CONFIRM anti-CD4 (SP35) Rabbit Monoclonal Primary Antibody **Roche Catalog #790-4423**
- ⊗ Anti-CD68 antibody mouse monoclonal [KP1] to CD68 **Abcam Catalog #(ab955)**
- ⊗ Anti-FOXP3 antibody mouse monoclonal [236A/E7] **Abcam Catalog #(ab20034)**
- ⊗ Anti-PD1 antibody [NAT105] (ab52587) Mouse monoclonal **Abcam Catalog #(ab52587)**
- ⊗ CD8 (Concentrate) Monoclonal Mouse Anti-Human CD8 Clone C8/144B **Agilent Technologies Catalog #M710301-2**
- ⊗ CD21 (2G9) Mouse Monoclonal Antibody **Cell Marque Catalog #121M-14**
- ⊗ EZ Prep (10x) **Roche Catalog #950-102**
- ⊗ Opal 7-Color Automation IHC Kit 50 Slides **Akoya Biosciences Catalog #NEL821001KT**
- ⊗ DISCOVERY UltraMap anti-Ms HRP (RUO) **Roche Catalog #760-4313**
- ⊗ DISCOVERY UltraMap anti-Rb HRP (RUO) **Roche Catalog #760-4315**
- ⊗ DISCOVERY Inhibitor **Roche Catalog #760-4840**
- ⊗ DAPI **Thermo Fisher Scientific Catalog #62248**
- ⊗ Dulbecco's Phosphate Buffered Saline (PBS) **Catalog #D8537**

Equipment

Vectra 3.0 Automated Quantitative Pathology Imaging System, 200 Slide NAME

Microscope TYPE

Vectra BRAND

CLS142338 SKU

<https://www.perkinelmer.com/product/vectra-3-imaging-system-200-slide-cls142338> LINK



Equipment

DISCOVERY ULTRA IHC/ISH

NAME

Autostainer

TYPE

Ventana

BRAND

05 987 750 001 / N750-DISU-FS

SKU

<https://diagnostics.roche.com/global/en/products/instruments/discovery-ultra.html>^{LINK}



Equipment

Light Sensitive Dispenser

NAME

User-fillable/Prep Kits

TYPE

Roche

BRAND

760-205

SKU

<http://reagent-catalog.roche.com/product/1455?type=1858>^{LINK}

Troubleshooting

Building a spectra library

- 1 A spectral library is initially built using positive controls of follicular lymphoma tissue to enable unmixing the 6 fluorophore spectra and DAPI in the multi-plex experiment. To build the library, the spectrum of each individual fluorophore is acquired from single-plex experiments.

The single-plex experiments are run with the same parameters and positions as in the multiplex experiment; all the pre-treatment steps are kept but only one antibody and its fluorophore are applied in the same order as for the multi-plex.

The order of antibodies and corresponding fluorophores:

Table 1. Order of antibodies and corresponding fluorophores.

Posit ion	Anti bod y	Fluo roph ore
1	CD4	Opal 620
2	CD6 8	Opal 650
3	CD8	Opal 540
4	CD2 1	Opal 570
5	FOX P3	Opal 520
6	PD1	Opal 690
7	DAPI	

The single-plex experiments are described below. The antibodies and fluorophores can be either pipetted manually on the slide by interrupting the automated protocol or prepared in a dispenser and applied automatically. For the multi-plex experiment which lasts 17 hours, we suggest the use of dispensers, whereas the single-plexes can be done manually with more flexibility.

The dilutions of antibodies and fluorophores can be found in Tables 2 and 3, respectively and are the same for multi-plex and single-plex experiments.

1.1

6h

CD4/620 Single-plex**Deparaffinisation**

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

1st Cycle

- Warm up slide to 36°C
- Automatically apply 'one drop' of primary **CD4 antibody** (pre-diluted dispenser) and incubate for **16 minutes**
- Apply one drop of **Ultramap anti-Rb HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100µl of the **620** diluted with TSA and incubate for **16 minutes**

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

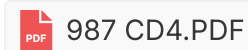
Remove the slides from the autostainer and wash with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

The automated protocol that should be programmed in the Ventana Discovery Ultra autostainer can be found here:



1.2

8h

CD68/650 Single-plex**Deparaffinisation**

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **CD68** and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100µm **650** diluted with TSA and incubate for **16 minutes**

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

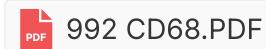
Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

**1.3 CD8/540 Single-plex**

8h

Deparaffinisation

In the autostainer:

Warm up slide to 69 °C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **CD8** and incubate for **32 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100 µm of diluted **540** and incubate for **16 minutes**

4th Cycle



- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning



1.4 CD21/570 Single-plex

8h

Deparaffinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **CD21** and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **24 minutes**
- Hand-pipette 100 µm of diluted **570** and incubate for **16 minutes**

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning



1.5 **FOXP3/520 Single-plex**

8h

Deparaffinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Hand-pipette 100 µm of diluted **FOXP3** and incubate for **60 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand-pipette 100 µm of diluted **520** and incubate for **16 minutes**

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and wash with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning



1.6 **PD1/690 Single-plex**

8h

Deparaffinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)
- Warm up slide to 36°C
- Hand - apply (pipette) 110µl of diluted **PD1** and incubate for **32 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand - apply 110µl of diluted **690** and incubate for **16 minutes**

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning



1.7 DAPI

5h

In this step the slide is run through the autostainer, without application of antibodies or fluorophores. Then it is removed, washed and DAPI is applied.

Deparaffinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

DAPI

Dilute DAPI 1:2000 with PBS, add one drop on the slides

Let rest in a dark slide box for 10 minutes

Rinse with TBST then water

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide

After coverslipping, let slides dry in a dark place for at least 2 hours before scanning



1.8 Autofluorescence

5h

The spectrum of autofluorescence can also be captured by running a slide through the autostainer deparafinisation and pretreatments without application of any antibodies and fluorophores, nor DAPI.

Deparafinisation

In the autostainer:

Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each

Pre-treatment and blocking

Warm up slide to 95°C and incubate for 40 minutes (CC1)

Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes

2nd Cycle

- Pre-treatment: Warm up slide to 95°C and incubate for **16 minutes** (CC1)

3rd Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

4th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

5th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

6th Cycle

- Pre-treatment: Warm up slide to 90°C and incubate for **8 minutes** (CC2)

Washing

Remove the slides from the autostainer and was with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

Coverslip

Two drops of prolong gold antifade reagent (without DAPI) is used per slide
After coverslipping, let slides dry in a dark place for at least 2 hours before scanning



2 Extracting the fluorophore spectra

Vectra 3.5 microscope is used to take snapshots of stained areas in the single-plex slides (withouth DAPI).

Snapshots are taken as multispectral images (MSI). Exposure times are set automatically for the filters relevant to each fluorophore. The filters where we do not expect to see signal from a fluorophore are set to the default value (150 ms for the MSI).

Expected result

When auto-exposing, the exposure times at 20x magnification should each be between 20-100ms for all filters. Higher exposure times would mean that the signal is in fact mostly autofluorescence. Lower exposure times could result in bleed through between the different markers in the multi-plex experiment. If exposure times need to be increased, the fluorophores can be diluted further to lower the signal's brightness.

Cells we see as positive for each fluorophore should appear only on the filters where we expect the fluorophore to emit. If these cells are seen in unexpected filters then they may be autofluorescing red blood cells instead of true positives, or the fluorophore might be too bright and should be diluted more.

InForm 2.4 software (PerkinELmer) is used to build the spectral library from these snapshots.


Expected result

When using the new spectral library to unmix the single-plex snapshots used to build it (and any other images) the signal to noise ratio, i.e. the intensity units on stained cells versus the intensity of the background, should be ideally more than 10:1, and at least 5:1.

Multiplex experiment

3 Multi-plex staining

17h

 1048 automated.PDF

The protocol is fully automated, except for the final steps, and lasts approximately 17 hours. For this reason we suggest running overnight and carrying out the final cycle (which is manual), washing, DAPI and coverslipping in the morning.

It is important that the single-plex experiments are satisfactory before the multi-plex experiments are started.

Preparation before starting

All antibodies and fluorophores will be pre-diluted and added in user fillable dispensers to be applied automatically by the autostainer. The dispensers can be used for at least a week following preparation (potentially longer but we have not tested this).

The following antibody dispensers are prepared by diluting the primary concentrates with the Antibody Diluent (included in the Opal kit). At least 1ml is added in each dispenser.

Table 2. Primary antibody dilutions. Antibodies are diluted with the antibody diluent included in the Opal kit.

Anti bod y	Volu me Rati o
CD4	Pre-dilut ed
CD8	1:45 0
CD6 8	1:40
FOX P3	1:60
CD2 1	1:25
PD1	1:150

Note

The PD1 antibody should only be used withing 2 days of diluting and preparing the dispenser. Thus the steps of the final cycle (6th) when PD1 is applied are preferably done by manually pipetting freshly prepared reagent onto the slide. Therefore no dispensers are prepared for PD1 and the corresponding Opal 690 fluorophore.

Light sensitive dispensers are preferably used for the fluorophores. The fluorophores are diluted with the Tyramide Signal Amplification liquid that is included in the Opal kit.

Table 2. Fluorophore dilutions. Fluorophores are diluted with the Tyramide Signal Amplification liquid included in the Opal kit.

Fluorophore	Fluorophore Dilution (Volume ratio)
Opal 520	1:75
Opal 540	1:150
Opal 570	1:60
Opal 620	1:150
Opal 650	1:75
Opal 690	1:75

Note

Caution: User fillable dispensers if prepared incorectly could dispense less or more than the nominal 100µl per 'drop'. Please refer to manufacturer's instructions.



- | | | |
|-----|--|--------|
| 3.1 | Deparaffinisation
In the autostainer:
Warm up slide to 69°C and incubate for 3 cycles of 8 minutes each | 24m |
| 3.2 | Pre-treatment and blocking
Warm up slide to 95°C and incubate for 40 minutes (CC1)
Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes | 44m |
| 3.3 | 1st Cycle <ul style="list-style-type: none">▪ Warm up slide to 36°C▪ Automatically apply 'one drop' of primary CD4 antibody (pre-diluted) and incubate for 16 minutes▪ Apply one drop of Ultramap anti-Rb HRP secondary and incubate for 16 minutes▪ Apply one drop of the 620 dispenser and incubate for 16 minutes | 2d |
| 3.4 | 2nd Cycle <ul style="list-style-type: none">▪ Pre-treatment: Warm up slide to 95°C and incubate for 16 minutes (CC1)▪ Warm up slide to 36°C▪ Apply one drop of CD68 dispenser and incubate for 60 minutes▪ Apply one drop of Ultramap anti-Ms HRP secondary and incubate for 16 minutes▪ Apply one drop of the 650 dispenser and incubate for 16 minutes | 1h 48m |
| 3.5 | 3rd Cycle <ul style="list-style-type: none">▪ Pre-treatment: Warm up slide to 90°C and incubate for 8 minutes (CC2)▪ Warm up slide to 36°C▪ Apply one drop of CD8 dispenser and incubate for 32 minutes▪ Apply one drop of Ultramap anti-Ms HRP secondary and incubate for 16 minutes▪ Apply one drop of the 540 dispenser and incubate for 16 minutes | 1h 12m |
| 3.6 | 4th Cycle <ul style="list-style-type: none">▪ Pre-treatment: Warm up slide to 90°C and incubate for 8 minutes (CC2)▪ Apply one drop of DISCOVERY Inhibitor and incubate for 4 minutes▪ Warm up slide to 36°C▪ Apply one drop of CD21 dispenser and incubate for 60 minutes▪ Apply one drop of Ultramap anti-Ms HRP secondary and incubate for 24 minutes▪ Apply one drop of the 570 dispenser and incubate for 16 minutes | 1h 48m |
| 3.7 | 5th Cycle <ul style="list-style-type: none">▪ Pre-treatment: Warm up slide to 90°C and incubate for 8 minutes (CC2)▪ Warm up slide to 36°C▪ Apply one drop of FOXP3 dispenser and incubate for 60 minutes▪ Apply one drop of Ultramap anti-Ms HRP secondary and incubate for 16 minutes▪ Apply one drop of the 520 dispenser and incubate for 16 minutes | 1h 40m |
| 3.8 | 6th Cycle <ul style="list-style-type: none">▪ Pre-treatment: Warm up slide to 90°C and incubate for 8 minutes (CC2) | 1h 12m |



- Warm up slide to 36°C
- Hand - apply (pipette) 110µl of diluted **PD1** and incubate for **32 minutes**
- Apply one drop of **Ultramap anti-Ms HRP** secondary and incubate for **16 minutes**
- Hand - apply 110µl of diluted **690** and incubate for **16 minutes**

3.9 **Washing**

Remove the slides from the autostainer and wash with EZ prep diluted 1:10 with distilled water (3 × 5 minutes).

15m

3.10 **DAPI**

Dilute DAPI 1:2000 with PBS, add one drop on the slides
Rest in a dark slide box for 10 minutes
Rinse with TBST then water

10m

3.11 **Coverslip**

Two drops of prolong gold antifade reagent (without DAPI) is used per slide
After coverslipping, let slides dry in a dark place for at least 2 hours before scanning

4 **Scanning and unmixing**

While scanning the multi-plex images all filters are auto-exposed in the Vectra microscope.

After scanning multispectrally at the same resolution as the single-plexes, the markers can be unmixed in inForm 2.4 using the spectral library.