Protocol for Revolune Illuminate-P4-R using BOND[1] RX

Instructions for Use

Product description

This kit is designed to perform single or multiplex immunofluorescence stainings of FFPE (Formalin-Fixed Paraffin-Embedded) samples.

The user can select up to four different rabbit IgG primary antibodies and need to pre-label them with 4 different Revolune Connectors (488, 555, 647 & 750). The staining protocol will be performed on the BOND[1] RX autostainer. The reagents are sufficient to stain 10 slides and the provided calculations are based on staining 10 slides in one run.

Product contents

Name	Quantity	Use
Revolune Amplifier A (4-plex)	1 mL	First amplification
Revolune Amplifier B (4-plex)	1 mL	First amplification
Revolune Amplifier C (4-plex)	1 mL	Second amplification and signal generation
Revolune Amplifier D (4-plex)	1 mL	Second amplification and signal generation
Revolune Connector 488 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolune Connector 555 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolune Connector 647 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolune Connector 750 (anti-rabbit IgG)	15 µL	Primary Ab labelling

Storage: All contents must be stored refrigerated (2-8°C). Revolune Amplifier C and D are light sensitive and the reagents should generally be stored in the dark.

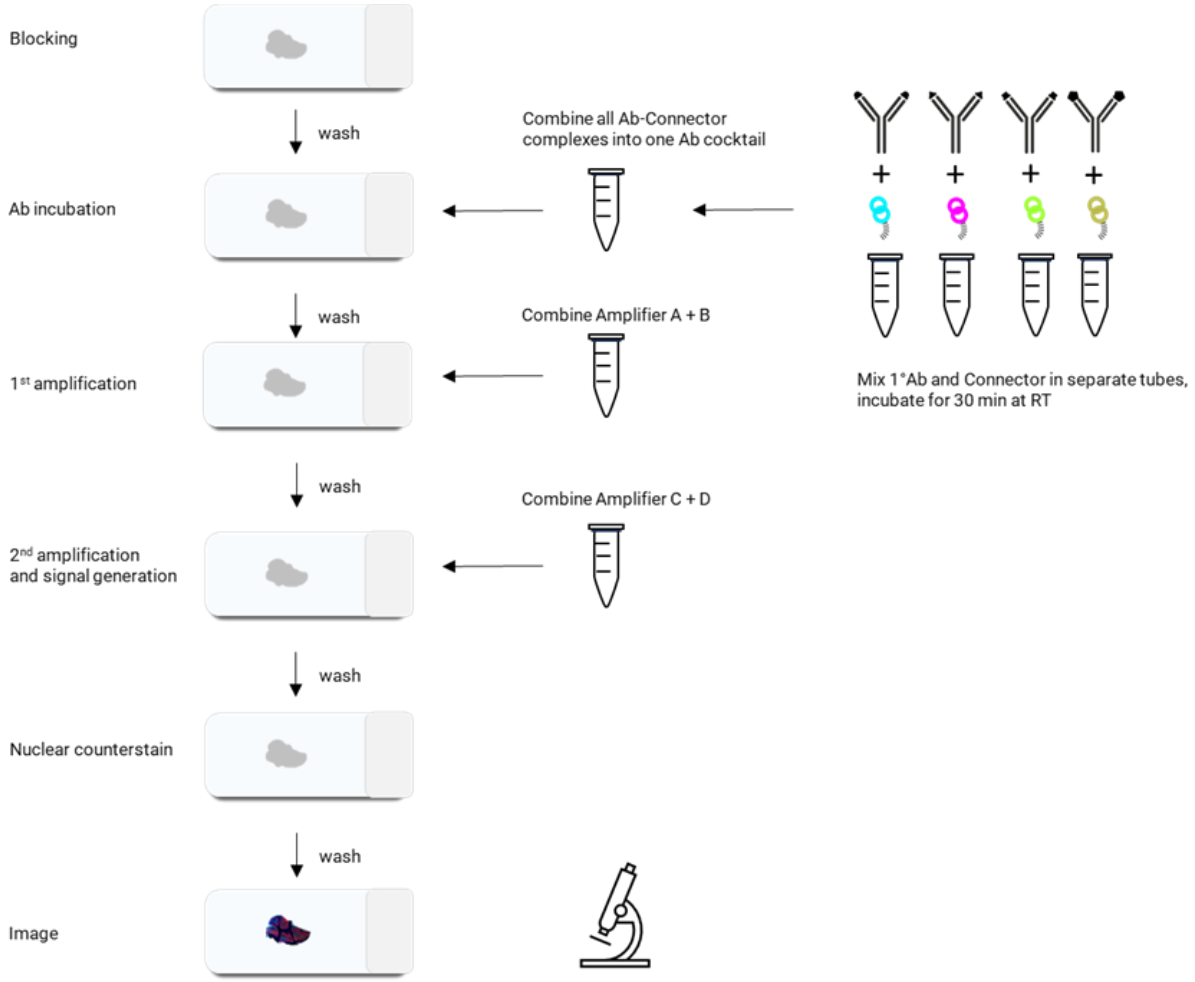
Reagents are stable for 3 months. Use within 4 weeks after opening.

Necessary reagents and consumables (not provided)

- BOND[1] primary antibody diluent, cat.no. AR9352
- BOND[1] wash solution 10X concentrate, cat. no. AR9590
- BOND[1] dewax solution, cat. no. AR9222
- BOND[1] epitope retrieval buffer 1, cat. no. AR9961
- BOND[1] epitope retrieval buffer 2, cat. no. AR9640
- BOND[1] research detection kit, cat.no. DS9455
- BOND[1] titration kit, cat.no. OPT9049

- Primary antibody (host species, isotype: rabbit, IgG)
- Ethanol (Histology-grade, >98%)
- 1x Phosphate Buffered Saline (PBS, pH 7.4)
- DAPI nuclear stain (working concentration 1 µg/mL)
- ProLong Gold Antifade mounting reagent (Invitrogen, cat.no. P36930). ***Note: Other mounting media may lead to rapid degradation of the signal***
- Standard lab supplies (pipettes/ tips, Eppendorf tubes)
- Glass coverslips

Overview of workflow



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Protocol

Part 1: Antibody preparation

In this step, the primary antibody (1° Ab) needs to be pre-labelled with Revolune Connectors. For calculation of the correct volumes, three variables (A,B,C) are needed:

- **A:** Stock concentration of 1° Ab [mg/mL]
- **B:** Working concentration of 1° Ab [µg/mL]
- **C:** Working solution volume [mL]

The stock concentration is typically provided by the antibody manufacturer.

The optimal working concentration of 1°Ab needs to be determined by the user. A good starting point is a concentration that works well for chromogenic IHC (e.g. DAB). Typical working concentrations are in the range of 0.2 µg/mL – 2 µg/mL.

The working solution volume [mL] depends on the number of slides to be stained. For BOND[1] RX, 150 µL antibody working solution is used per slide and the titration container has a dead volume of 300 µL. For 10 slides, a minimum of 1800 µL (10 x 150 µL + 300 µL) need to be prepared. However, we recommend to prepare 2000 µL to account for pipetting errors.

Based on the three variables A, B & C, the volume of 1° Ab (**D**) and volume of Revolune Connector (**E**) (5 µL per 1 µg 1°Ab) are calculated as follows:

$$D = (B \div A) \times C$$

$$E = B \times C \times 5 \frac{\mu L}{\mu g}$$

Calculation example: A = 1 mg/mL, B = 1 µg/mL, C = 2 mL

$$D = 1 \frac{\mu g}{mL} \div 1 \frac{mg}{mL} \times 2\, mL = 2\, \mu L$$

$$E = 1 \frac{\mu g}{mL} \times 2\, mL \times 5 \frac{\mu L}{\mu g} = 10\, \mu L$$

Each 1° Ab needs to be labelled with a different Revolune Connector. **It is essential to use the correct amount of Revolune Connectors to saturate all binding sites on the 1° Ab to avoid cross-talk in a multiplex experiment**. For example, 1° Ab against target 1 is labelled with Revolune Connector 488 (488 channel), 1° Ab against target 2 with Revolune Connector 555 (555 channel), etc.

The labelling should be performed in separate reaction tubes. Please start with 10 µL PBS and add suitable amounts of 1° Ab and Revolune Connector. The following table shows an example calculation for staining 10 slides (using 2000 µL working solution), for various concentrations:

Primary antibody target	Primary Ab labelling reagent	A: Stock conc. 1° Ab [mg/mL] **(A)**	Working conc. 1° Ab [µg/mL] **(B)**	Working solution volume [mL] **(C)**	PBS	µL needed of 1° Ab **(D)**	µL needed of Revolune Connector **(E)**
Target 1	Revolune Connector 488	1.0	1.0	2	10 µL	2 µL	10 µL
Target 2	Revolune Connector 555	1.0	1.5	2	10 µL	3 µL	15 µL
Target 3	Revolune Connector 647	0.5	0.5	2	10 µL	2 µL	5 µL
Target 4	Revolune Connector 750	0.5	1.0	2	10 µL	4 µL	10 µL

The following formula and table may be used as template to calculate the amounts for your assay:

$D = (B \div A) \times C$

$E = B \times C \times 5 \frac{\mu L}{\mu g}$

Primary antibody target	Primary Ab labelling reagent	Stock conc. 1° Ab [mg/mL] **(A)**	Working conc. 1° Ab [µg/mL] **(B)**	Working solution volume [mL] **(C)**	PBS	µL needed of 1° Ab **(D)**	µL needed of Revolune Connector **(E)**
	Revolune Connector 488				10 µL		
	Revolune Connector 555				10 µL		
	Revolune Connector 647				10 µL		
	Revolune Connector 750				10 µL		

Mix PBS, 1° Ab and Revolune Connectors in four separate reaction tubes gently by flicking the tube, spin down the vials briefly and incubate for 30 min at room temperature (RT). The 1°Ab are now ready for the amplification step.

Part 2: BOND[1] reagent container preparation

Use a BOND[1] research detection kit (DS9455) with wash buffer in position 1 and five titration kit containers (OPT9049). One container will be used as MARKER and four containers (OPEN1, 2, 3 & 4) for staining reagents. All reagents need to be prepared freshly before the run. The following protocol gives instructions to stain 10 slides (volumes can be adjusted for different staining volumes):

1) Mix the four Ab-Connector complexes (Part 1) with BOND[1] primary antibody diluent (AR9352) for a total volume of 2 mL. This will be used as MARKER in the BOND[1] protocol.
2) Prepare a titration container with 2 mL BOND[1] primary antibody diluent (AR9352). This will be used as OPEN1 (Blocking).
3) Prepare a titration container with 1 mL Revolune Amplifier A and 1 mL Revolune Amplifier B. This will be used as OPEN2 (First amplification reaction)
4) Prepare a titration container with 1 mL Revolune Amplifier C and 1 mL Revolune Amplifier D. This will be used as OPEN3 (Second amplification reaction and labelling)
5) Prepare a titration container with 3.5 mL DAPI counterstain, diluted in BOND[1] wash buffer (DO NOT use water or other diluents). This will be used as OPEN4. We recommend using DAPI at 1 µg/mL. If the stock solution is 1 mg/mL, dilute 3.5 µL DAPI stock solution in 3.5 mL BOND[1] wash buffer.The counterstain is applied two times per sample (step 17 and 18 in the BOND[1] protocol).

Overview of prepared BOND[1] titration container to stain 10 slides:

Name	Reagent	Reagent volume [µL]
MARKER	Connector-labelled antibodies	2000
OPEN1	Blocking (ab dilution buffer)	2000
OPEN2	Revolune Amplifier A + Revolune Amplifier B	1000 (A) + 1000 (B)
OPEN3	Revolune Amplifier C + Revolune Amplifier D	1000 (C) + 1000 (D)
OPEN4	DAPI (1 µg/mL)	3500

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Part 3: BOND[1] protocol setup

Slide preparation: Dewax and HIER (e.g. ER2, 20 min). This can be altered based on the used antibodies and established lab procedures.

Set up a protocol on BOND[1] RX (staining method: single) with the following parameters:

Step	Reagent	Incubation time [min]	Temperature	Dispense volume [µL]
1	Wash buffer (Research Detection kit)	0:00	RT	150
2	*BOND[1] wash solution	0:00	RT	150
3	*BOND[1] wash solution	0:00	RT	150
4	OPEN1	15:00	RT	150
5	MARKER	60:00	RT	150
6	*BOND[1] wash solution	1:00	RT	150
7	*BOND[1] wash solution	1:00	RT	150
8	*BOND[1] wash solution	1:00	RT	150
9	OPEN2	90:00	RT	150
10	*BOND[1] wash solution	1:00	RT	150
11	*BOND[1] wash solution	1:00	RT	150
12	*BOND[1] wash solution	1:00	RT	150
13	OPEN3	90:00	RT	150
14	*BOND[1] wash solution	1:00	RT	150
15	*BOND[1] wash solution	1:00	RT	150
16	*BOND[1] wash solution	1:00	RT	150
17	OPEN4	5:00	RT	150
18	OPEN4	5:00	RT	150
19	*BOND[1] wash solution	1:00	RT	150
20	*BOND[1] wash solution	1:00	RT	150
21	*BOND[1] wash solution	1:00	RT	150
22	*BOND[1] wash solution	1:00	RT	150

Protocol run time (without Dewax and HIER): ca. 4h 45min

Part 4: Mounting and storage

1) After the BOND[1] run, remove any excess liquid from the specimen by tapping the slide on to a clean tissue. **Do not wash the slide with water.**
2) Place a drop or suitable amount (e.g. 50 µL) ProLong Gold onto a clean coverslip and carefully lower it onto the specimen. *Note: Other mounting media may lead to rapid degradation of the signal.*
3) Allow the mounted slide to cure on a flat surface in the dark at room temperature. Curing time may vary from a couple of hours to overnight.
4) Image the samples as soon as possible after curing for best fluorescent signal.
5) Store the samples at 4°C to best preserve the signal. It is stable for at least 1 week. Storing at room temperature is not recommended.

[1] BOND is a trademark of Leica Biosystems.