

Protocol for Revolute Illuminate-P7-R using BOND¹ 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 seven different rabbit IgG primary antibodies and need to pre-label them with seven different Revolute Connectors (430, 480L, 488, 555, 594, 647 & 750). The staining protocol will be performed on the BOND¹ 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
Revolute Amplifier A (7-plex)	1 mL	First amplification
Revolute Amplifier B (7-plex)	1 mL	First amplification
Revolute Amplifier C (7-plex)	1 mL	Second amplification and signal generation
Revolute Amplifier D (7-plex)	1 mL	Second amplification and signal generation
Revolute Connector 430 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolute Connector 480L (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolute Connector 488 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolute Connector 555 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolute Connector 594 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolute Connector 647 (anti-rabbit IgG)	15 µL	Primary Ab labelling
Revolute Connector 750 (anti-rabbit IgG)	15 µL	Primary Ab labelling

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

Reagents are stable for 6 months. Use within 4 weeks after opening.

Necessary reagents and consumables (not provided)

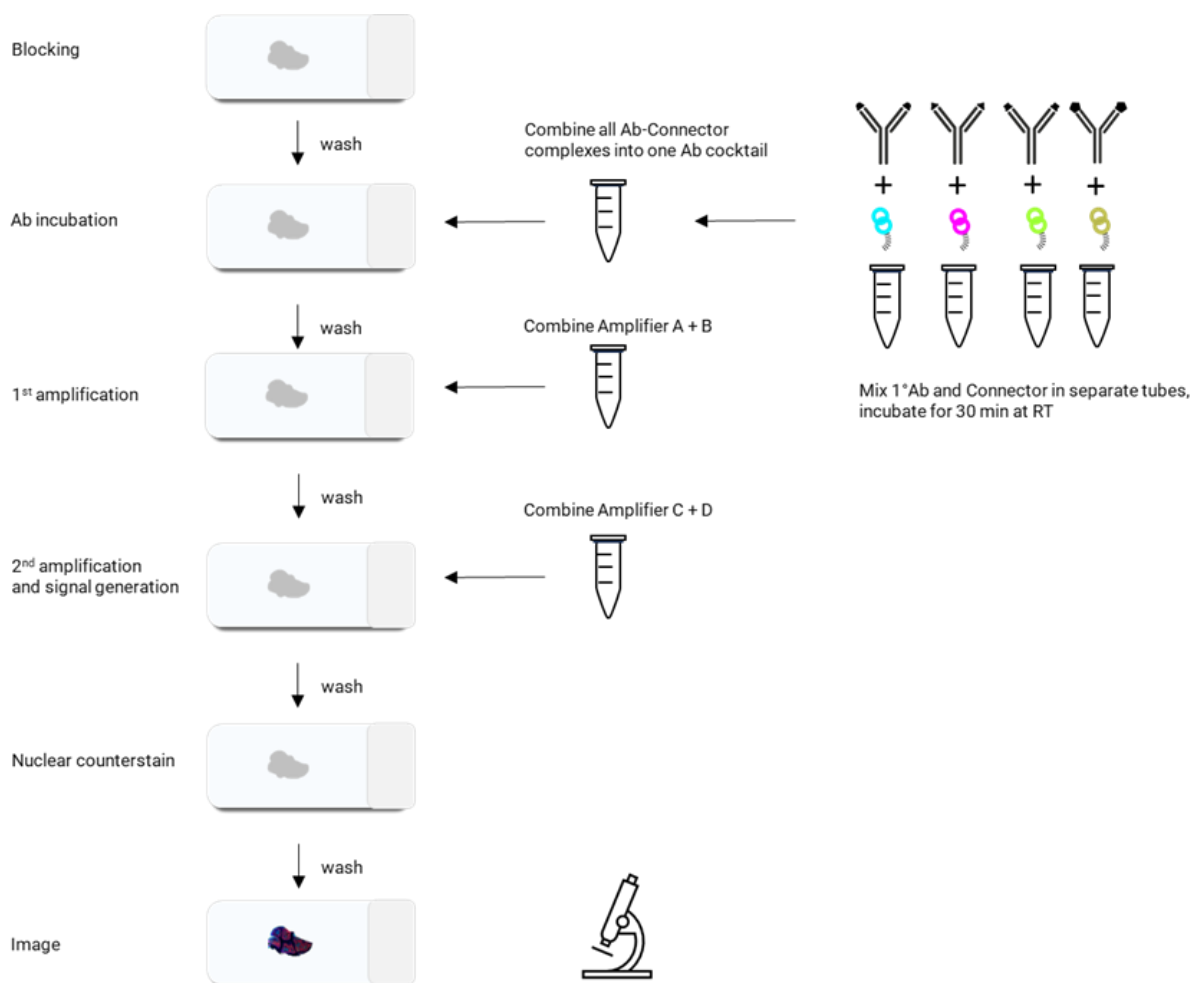
- BOND¹ primary antibody diluent, cat.no. AR9352
- BOND¹ wash solution 10X concentrate, cat. no. AR9590
- BOND¹ dewax solution, cat. no. AR9222

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- BOND¹ epitope retrieval buffer 1, cat. no. AR9961
- BOND¹ epitope retrieval buffer 2, cat. no. AR9640
- BOND¹ research detection kit, cat.no. DS9455
- BOND¹ 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 Revolute 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 BOND¹ 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 Revolute 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

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

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

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Each 1° Ab needs to be labelled with a different Revolute Connector. **It is essential to use the correct amount of Revolute 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 Revolute Connector 488 (488 channel), 1° Ab against target 2 with Revolute 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 Revolute 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 Revolute Connector (E)
Target 1	Revolute Connector 430	1	0.5	2	10 µL	1 µL	5 µL
Target 2	Revolute Connector 480L	1	0.75	2	10 µL	1.5 µL	7.5 µL
Target 3	Revolute Connector 488	1.0	1.0	2	10 µL	2 µL	10 µL
Target 4	Revolute Connector 555	1.0	1.5	2	10 µL	3 µL	15 µL
Target 5	Revolute Connector 594	1.0	1.5	2	10 µL	3 µL	15 µL
Target 6	Revolute Connector 647	0.5	0.5	2	10 µL	2 µL	5 µL
Target 7	Revolute Connector 750	0.5	1.0	2	10 µL	4 µL	10 µL

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

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

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

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 Revolute Connector (E)
	Revolute Connector 430				10 μL		
	Revolute Connector 480L				10 μL		
	Revolute Connector 488				10 μL		
	Revolute Connector 555				10 μL		
	Revolute Connector 594				10 μL		
	Revolute Connector 647				10 μL		
	Revolute Connector 750				10 μL		

*We recommend using 10 μL PBS to facilitate pipetting of small volumes and mixing. This 10 μL is independent of the actual slide number used.

Mix PBS, 1° Ab and Revolute Connectors in seven 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.

You can also use our Reagent Calculator tool on the website: [Calculator](https://www.revolute.de/support) (https://www.revolute.de/support).

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Part 2: BOND¹ reagent container preparation

Use a BOND¹ 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.** We don't recommend a delayed start. The following protocol gives instructions to stain 10 slides (volumes can be adjusted for different staining volumes):

- 1) Mix the seven Ab-Connector complexes (Part 1) with BOND¹ primary antibody diluent (AR9352) for a total volume of 2 mL to create your antibody cocktail mix. This will be used as MARKER in the BOND¹ protocol.
- 2) Prepare a titration container with 2 mL BOND¹ primary antibody diluent (AR9352). This will be used as OPEN1 (Blocking).
- 3) Prepare a titration container with 1 mL Revolute Amplifier A and 1 mL Revolute Amplifier B. This will be used as OPEN2 (First amplification reaction)
- 4) Prepare a titration container with 1 mL Revolute Amplifier C and 1 mL Revolute 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 BOND¹ 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 BOND¹ wash buffer. The counterstain is applied two times per sample (step 17 and 18 in the BOND¹ protocol).

Overview of prepared BOND¹ titration container to stain 10 slides:

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

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Part 3: BOND¹ 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 BOND¹ 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 BOND¹ 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.