

## EXPEDITING MXIF PANEL DEVELOPMENT IN FFPE FOR THE PHENOCYCLER PLATFORM: THE 'JOKER' PANEL DEVELOPMENT KIT

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**Background** Highly multiplexed cyclic immunofluorescence (MxIF) platforms, such as the PhenoCycler system (formerly CODEX) by Akoya Biosciences allow users to stain and visualize up to 100 markers on a single formalin-fixed paraffin-embedded (FFPE) tissue section. The PhenoCycler accomplishes this feat by staining the tissue with antibodies that have been conjugated to a unique single-stranded oligonucleotide barcode (BX). Reporters (RX), containing the complimentary sequence and tagged with one of 3 spectrally-resolved fluorophores, are subsequently added, imaged and then gently removed via isothermal reaction.<sup>1</sup> With the advent of this type of technology, comes the opportunity to apply it to the study of diverse tissues and biological conditions. However, in this pursuit, investigators are often faced with the challenge of developing custom reagents for the platform. Factors such as tissue autofluorescence, antigen density, cell density, fluorophore signal sensitivity, and conjugation efficiency need to be considered to optimize panel development.

**Methods** To increase probability of success, expedite panel development, and optimize reagent utilization, we have developed the 'Joker' panel development kit. The 'Joker' kit consists of (1) BX007, (2) three versions of RX007, each conjugated to one of the 3 antibody-reading channels (AlexaFluor®750, Atto550, and AlexaFluor®647), and (3) a tissue microarray (TMA) containing 16 unique human tissues. As proof of concept, we conjugated a previously validated CD45 monoclonal antibody to our 'Joker' BX007.

**Results** Conjugation of CD45 to BX007 was verified via flow cytometry and by tissue using the 'Joker' TMA (figure 1A,B). Cycles in which no markers were revealed provided

information on backgrounds present in different tissue types (figure 2A). A representative immune cell for each tissue was identified and pixel intensity was calculated for CD45-BX007 with respect to background (table 1). We demonstrated that this kit could be used to quickly screen custom markers across tissue types in a single run. We show that CD45-BX007 is compatible with all channels over multiple tissues (figure 2A, B).

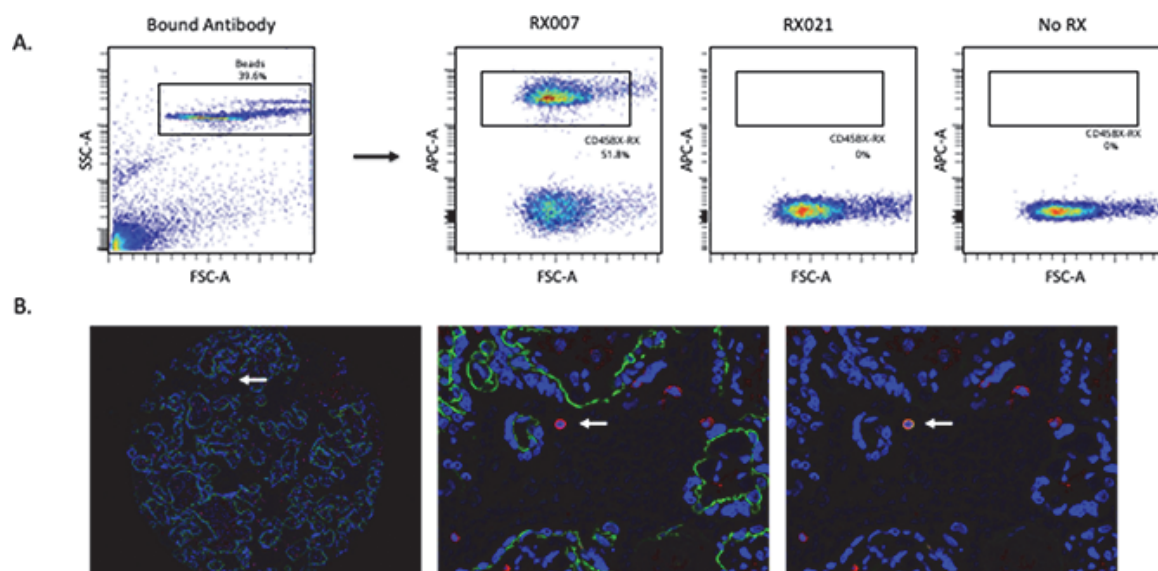
**Conclusions** Utilizing the 'Joker' kit we can expedite determination of the best channel-tissue combination for any given custom-developed antibody under the biological conditions we seek to study. This is critical for MxIF panel development and especially important for low expression markers that may give out false positive results if acquired on channels with high background. Alternatively, false negative results may occur if the antibody is visualized on a low sensitivity channel. The 'Joker' kit can greatly facilitate the panel development effort of researchers using the PhenoCycler MxIF system.

### REFERENCE

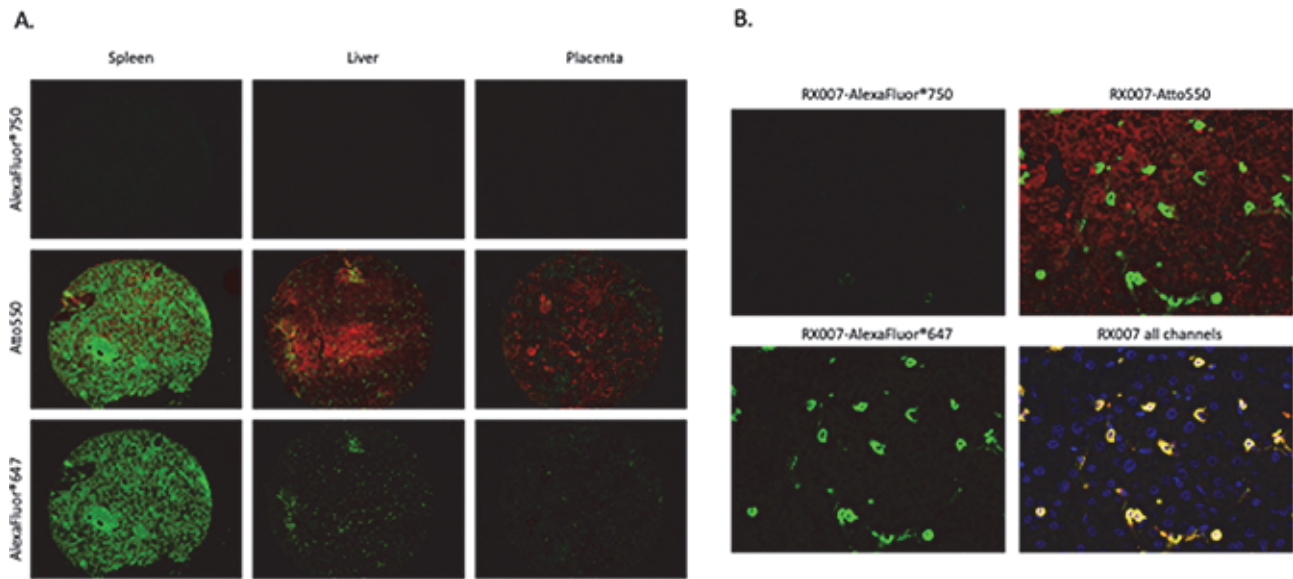
- Black, Sarah, *et al.* 'CODEX multiplexed tissue imaging with DNA-conjugated antibodies.' *Nature protocols.* 2021;**16**(8):3802–3835.

**Abstract 191 Table 1** Background and Signal intensities all acquisition channels for CD45-BX007 on a sampling of tissues from the 'Joker' TMA. Atto550 is the highest background channel, where as AlexaFluor\*647 has the lowest background. Atto550 should be avoided for highly auto fluorescent tissue and/or low expressing markers.

	Background AlexaFluor*750	Background Atto550	Background AlexaFluor*647	RX007-AlexaFluor*750	RX007-Atto550	RX007-AlexaFluor*647	SNR of RX007-AlexaFluor*647
Spleen	190	596	284	1484	25815	23364	82.2
Liver	175	3488	474	588	32507	23572	49.7
Placenta	180	3806	585	282	15971	23205	39.4



**Abstract 191 Figure 1** Confirmation of successful conjugation of CD45 to 'Joker' BX007. (A) Conjugation tested by flow cytometry using compensation beads, CD45-BX007, and RX spike in. RX on AlexaFluor\*647 used. No signal present when incorrect BX-RX pair (RX021) used, or no RX is added. (B) 'Joker' TMA stained with CD45-BX007, along with a small panel of co- and counter expression markers to verify staining. Above shows a placenta core present on the TMA. Counter localization of CD45 (red) and E-cadherin (green) shown in middle panel. Colocalization of CD45 (red) with CD3 (green) show in yellow in the right most panel. Verifying conjugation via tissue staining is necessary in order to demonstrate the conjugation process did not alter antibody affinity or specificity.



**Abstract 191 Figure 2** Evaluation of channel-tissue combination through use of "Joker" Panel Development Kit. (A) Signal for background (red) and CD45 (green) thresholded equally across channel and marker on various tissues present on the "Joker" TMA. Knowledge of channel sensitivity, tissue background, and marker signal across various tissues is gained from a single acquisition and can be used to determine the optimal channel-antibody pairing. (B) Single cell look at CD45 positive cells (green) in liver revealed across all three acquisition channels. Background for each channel is included in red. Although CD45 is detectable across all channels, sensitivity varies. This knowledge can also be used to reserve AlexaFluor\*647 for conjugation to markers with low expression or antigen density. High expressing markers, such as CD45 in lymphoid tissues, are able to tolerate higher background, low sensitivity channels (Atto550 or AlexaFluor\*750) and therefore are more flexible when it comes to panel design. Bottom right image shows colocalization of CD45 positive cells across the three channels and DAPI.

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