Antibody Screening and Custom-conjugation - Tips and guidelines

Selecting antibody clones:

- CODEX® inventoried and CODEX® screened antibodies:

  Apart from the commercially available CODEX® antibodies, Akoya has a list of non-inventoried CODEX® screened antibodies that have been shown to work. The list provides information on the clone, vendor, tested tissue, and antigen retrieval conditions.
  The user can select clone(s) from this list, test it on their tissue of interest, and confirm that the clone suits their needs.

- If Akoya hasn't tested the marker:

  If possible, work with clones that are known to work on the tissue of interest with IHC – based on experience, literature, etc. Additionally, we recommend working with IgG isotypes preferentially. IgM clones may have a higher failure rate.

Validate unconjugated clone:

- Staining conditions:

  Before performing the conjugation with CODEX® barcodes, it is critical to identify the best-suited antibody clone and optimize the staining conditions, like antigen retrieval, using the unconjugated antibody clone and the positive tissue. This can be performed using standard IF or IHC and the positive test tissue

- Specificity:

  At this point, the user may also consider assessing the specificity of the antibody clone. This can be done by staining with the antibody clone and a positive and negative counterstain when possible.

  Note: When working with rare markers, please ensure to screen for positive tissue using standard IF or IHC

Use Purified Antibody clones:

- Work with pre-purified antibodies if possible:

  When selecting clones for conjugation to CODEX® barcodes, consider purchasing purified antibodies, in PBS or a similar buffer, that is free of carrier proteins, such as BSA, gluten, glycerol etc. and other such additives.

- Purify before conjugation:

  If purified clones are not commercially available, a purification process must be performed before conjugation. We have recommendations for purification kits.

- Quantify antibodies accurately:

  Once any purification is performed, the concentration of the purified antibody must be measured again to ensure 50 ug are present using Nanodrop or a similar tool.

  Please note:
  1)The concentrations presented on the labels of commercial antibodies are usually not accurate, and they are likely to lead to poor conjugation efficiencies.

  2) The process of any antibody purification and conjugation, in general, involves washing and purification steps which can result in a reduction of the antibody concentration and hence it is critical to start with the recommended 50 ug of purified antibody.

Selecting CODEX® barcodes:

- Low abundance antigens:

  When choosing CODEX® barcodes for custom-conjugation, please consider that less abundant antigens produce low-intensity signals and perform better if conjugated to CODEX® barcodes assigned to fluorescence channels with low autofluorescence (the corresponding reporter dyes are Cy5 and ATTO550 for fresh-frozen tissues, and Cy5 for FFPE).

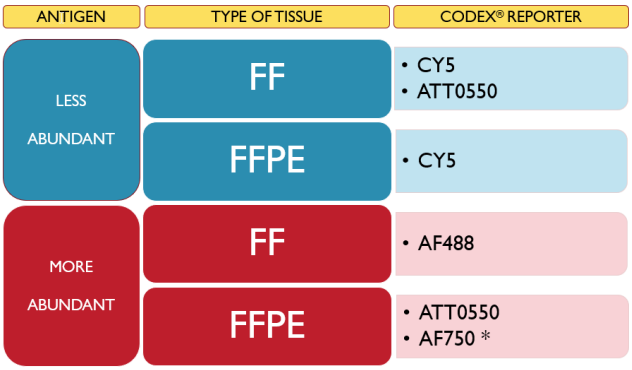
- High abundance antigens:

  Correspondingly, for antibodies targeting highly expressed antigens, we recommend using CODEX® barcodes corresponding to AF488 (for fresh-frozen tissues), and ATTO550 and AF750 (for FFPE tissues).

  These channels are recommended only for highly expressed antigens because of:
  1) high autofluorescence (AF488 for fresh-frozen and ATTO550 for FFPE)
  2) Suboptimal camera sensitivity (AF750).

- Scheme for assigning CODEX® barcodes to specific antibodies

  Users can follow the scheme below for assigning barcodes to different antibodies.



*After preliminary screening with ATTO 550 or CY5

Custom-conjugated Antibody validation workflow:

Antibody validation is critical to ensure accurate and reproducible results. Commercial CODEX® tagged antibodies are already validated based on Akoya's rigorous validation scheme. We recommend that for each new batch of custom-conjugated CODEX® barcoded antibodies, the researcher performs appropriate validation experiments.

Note: Presented here is the Akoya recommended validation workflow scheme. User may adopt and adjust the workflow as per the rigor necessary for their experimental setup

Tissue selection for antibody screening:

1. Identify positive tissue for staining – a tissue that expresses the target of interest

2. Identify negative tissue for staining – a tissue that does not express the target of interest

Note: It is highly recommended to image unstained tissue sections using each fluorescent channel of interest before staining. This will allow identifying tissues/tissue regions with least autofluorescence.

Custom-conjugation:

Perform Antibody conjugation using the selected antibody clone and CODEX® Barcodes as described in the antibody conjugation protocol (Refer to CODEX® user manual for the custom-conjugation protocol)

Note: Please work with purified antibody in PBS. Perform conjugation as recommended in the CODEX® user manual. Confirm the conjugation by gel electrophoresis first, before performing the staining experiments.

Single-stain validation:

A. Specificity:

The validation of conjugated antibodies is only complete once the antibodies are used in a staining experiment. Perform single stains using positive and negative tissue.

a. It is recommended to work with three serial sections of the positive tissue:

   1. CODEX® tagged Antibody stain

   2. CODEX® tagged antibody stain with Costain (dye-conjugated positive control antibody that targets an antigen expressed by the same population of cells)

   3. CODEX® tagged antibody stain with counterstain (dye-conjugated negative control antibody that targets an antigen expressed by a distinct/different population of cells)

b. Please make sure that the selected control antibodies (dye-conjugated antibodies) emit in a different channel with respect to the CODEX®-tagged Antibody.
c. Please use the same fluorescence microscope with the same filter cubes, camera settings, lamp/LED intensity, etc. for the single stain validations that one would for CODEX® runs.
d. Calculate/Observe the degree of co-staining between the CODEX®-tagged antibody and the Positive Control (the higher, the better).
e. Calculate/Observe the degree of overlap between the CODEX®-tagged antibody and the Negative Control (the lower, the better).
f. Make sure that the fluorescence intensity and SNR from the CODEX®-tagged antibody (obtained as an average of the values corresponding to the three different tissues) are satisfactory.

B. Antibody titer:

For every new batch of conjugated antibody clone, a titration experiment has to be performed to find the best working concentration (usually titrated using a positive test tissue across antibody concentrations to find the concentration that provides the highest sensitivity, specificity, and Signal to Noise Ratio (SNR)

Recommended starting titration for testing:

For FF : 1:250

For FFPE : 1:50

Note: The titration dilutions can also be decided based on the staining observed using the unconjugated clone. User will find that a higher concentration will have to be used post-conjugation, as the process of conjugation is not 100% efficient and results in loss of antibodies.

Multicycle run:

Lastly, it is critical to confirm that the antibody stain works in a multicycle run at the single stain validated concentration. This ensures that the staining is unaltered by the experimental conditions of a CODEX® run.