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Efficient Discovery of Target-Specific Antibodies Using High-Content Imaging

OVERVIEW

Cell Signaling Technology (CST) uses a rigorous set of validation strategies to ensure that antibodies developed are highly specific and sensitive.

- To discover and develop specific antibodies with utility in multiple applications, it is critical to assess samples throughout the development process using these desired applications.
- Antibodies intended for use in cell-based immunofluorescence assays (IF) are evaluated using High-Content Imaging (HCI) across multiple stages of development, from primary screens through final product formulation (Diagram 1).
- A commonly used tool for high-throughput antibody screening is ELISA, however it is not a good measure for prediction of utility in IF.
- At CST, HCI is performed alongside ELISA during antibody discovery because it enables antibody screening using a high-throughput immunofluorescence protocol.
- Using HCI as an antibody discovery and validation tool has enabled the generation of hundreds of highly specific antibodies with utility in IF and led to the development of a broad portfolio of antibodies that can be used in HCI.

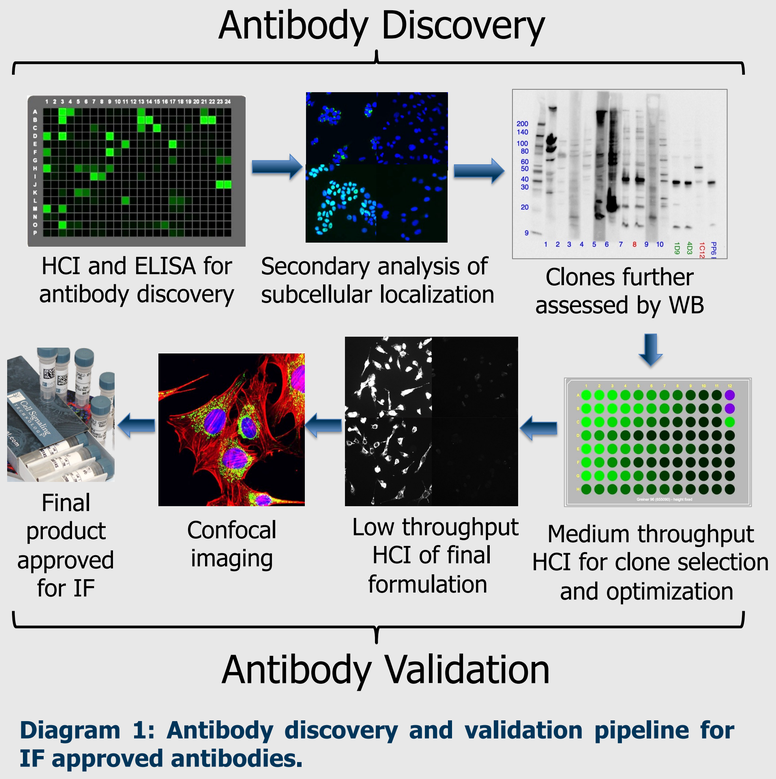

Diagram 1: Antibody discovery and validation pipeline for IF approved antibodies.

METHODS

- Supernatant from immortalized antibody-producing cells was tested in parallel by HCI and ELISA.
- Supernatant was added to cell lines containing the target of interest (+) and cell lines null for the target of interest (-).
- Supernatant samples were evaluated using the CST Immunofluorescence Protocol.
- Quantitative data analysis was performed to identify lead candidates.
  - Mean fluorescence intensity (MFI) was quantified using an SPT Labtech Acumen Explorer eX3.
  - Signal-to-noise ratio (S/N) was calculated by dividing the MFI in + cells by the MFI in - cells.
- Secondary analysis was performed to further characterize hits by evaluating subcellular localization of bound antibody.
  - Images captured by widefield, high-resolution imaging using a Thermo Fisher Scientific Cellomics ArrayScan VTI.
- Antibody specificity of samples characterized as hits by HCI and ELISA was further assessed by Western Blot assay (WB).
- Lead candidate clones were recombinantly expressed and retested using HCI and high-resolution confocal microscopy.

RESULTS

1. Quantitative Data Analysis Identifies Lead Candidates

- Analysis of S/N fluorescence ratios enables elimination of undesired antibodies, characterized by lack of binding or non-specific binding (S/N values close to 1).
- Samples that generate S/N fluorescence ratios greater than 2 are taken through secondary analysis.
- 17 samples in the ITM2A screen (Fig 1a) and 59 samples in the ZHX2 screen (Fig 1b) were chosen for secondary analysis.
- Blue and red dots indicate samples that exhibited correct localization after secondary analysis (blue: hits identified by ELISA and HCI, red: hits identified by HCI only).

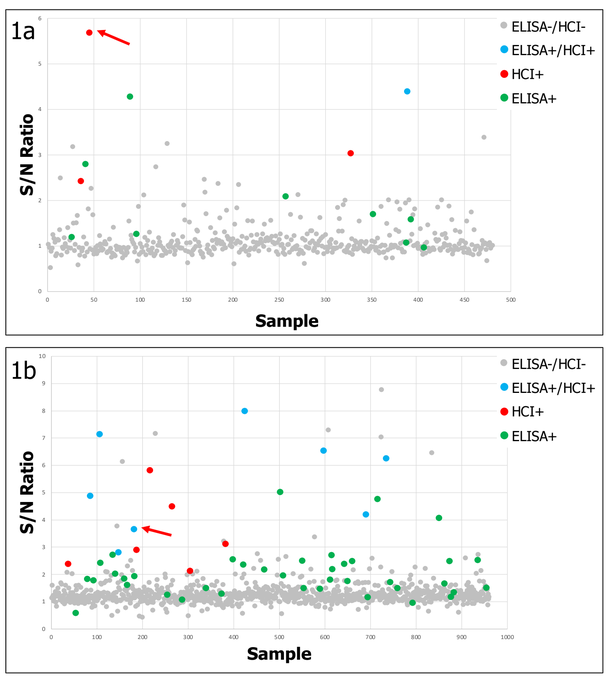

Figure 1: S/N Analysis and Overlap with ELISA
S/N analysis after HCI and overlap with ELISA for protein target ITM2A (a) and ZHX2 (b) (hits identified by ELISA and HCI: blue, hits identified by HCI only: red, hits identified by ELISA only: green, samples not identified as hits: grey). Red arrows indicate samples that were validated using HCI and high-resolution confocal microscopy.

2. Secondary Analysis Evaluates Subcellular Localization

- Evaluation of subcellular localization enables elimination of antibodies that do not bind the target protein in the expected localization.
- ITM2A antibody bound ITM2A in the Golgi apparatus (Fig 2a). No antibody bound in ITM2A-null cells (Fig 2b).
- ZHX2 antibody bound ZHX2 in nuclei (Fig 2c). No antibody bound in ZHX2-null cells (Fig 2d).
- 4 samples in the ITM2A screen (Fig 1a; red, blue) and 14 samples in the ZHX2 screen (Fig 1b; red, blue) were characterized as hits by HCI after secondary analysis.

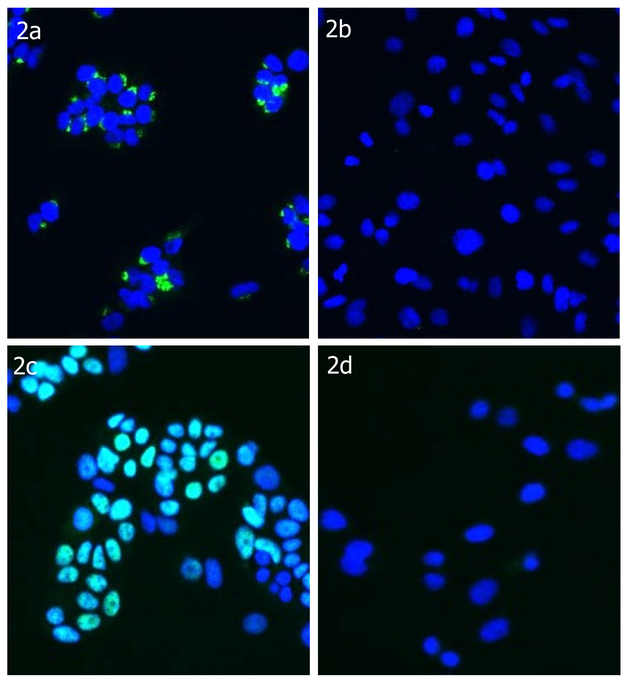

Figure 2: Secondary Analysis Using High-Resolution Imaging
High-resolution imaging of ITM2A antibody in A-673 cells (a) and Hep G2 cells (b) and ZHX2 antibody in MCF7 cells (c) and AGS cells (d) (blue: nuclear dye, green: antibody).

3. Western Blot Further Assesses Specificity

- Samples that were identified as hits by HCI also bound protein at the correct molecular weight in WB assays.
- In the ITM2A screen, only samples identified as hits by HCI exhibited correct performance by WB. ELISA activity did not translate to WB functionality (Fig 3a).
- In the ZHX2 screen, samples identified as hits by both ELISA and HCI exhibited correct WB activity, however pursuing any samples selected by ELISA only would result in an antibody product that would not be suitable for use in IF (Fig 3b).
- Red arrows indicate samples recombinantly expressed and validated using HCI and high-resolution confocal microscopy.

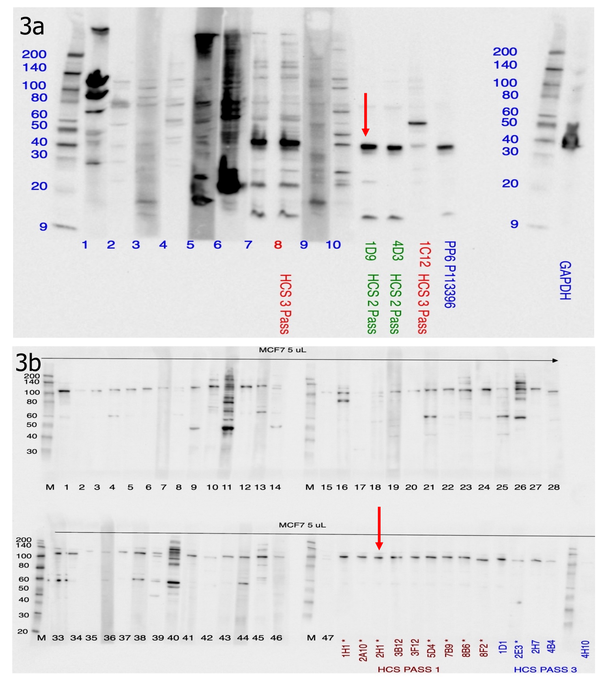

Figure 3: Western Blot Analysis of Hits
WB analysis of anti-ITM2A samples characterized as hits by ELISA (blue) and HCI (red, green) (a) and anti-ZHX2 samples characterized as hits by ELISA (black) and HCI (brown, blue) (b). ELISA and HCI overlapping hits (*).

4. Confocal Immunofluorescent Analysis Confirms Antibody Specificity and Utility in IF

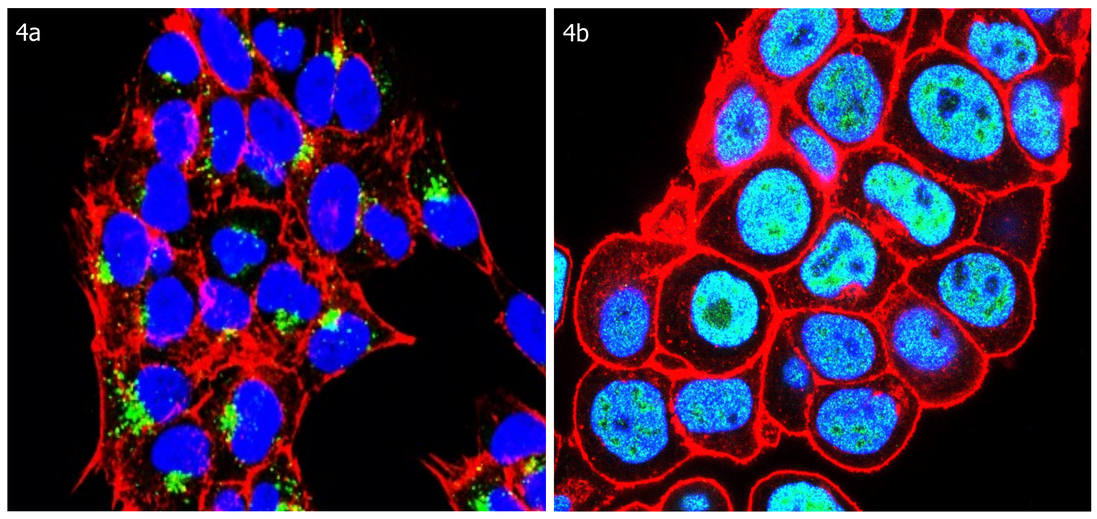

Figure 4: Confocal Immunofluorescent Analysis
Prior to antibody product release, imaging by confocal microscopy confirms antibody specificity and utility in IF. Confocal immunofluorescent analysis of ITM2A (1D9_E7G8A) Rabbit mAb (#73561, green) (a) and ZHX2 (2H1_E5H5G) Rabbit mAb (#43035, green) (b). Anti-ITM2A binds ITM2A in the Golgi apparatus as expected. Anti-ZHX2 binds ZHX2 in nuclei as expected.

CONCLUSIONS

- HCI is a powerful tool that can be used to supplement ELISA for antibody discovery.
  - HCI enables discovery of antibodies that are functional in cell-based IF that might not be identified using a peptide-based assay like ELISA alone.
  - HCI enables discovery of a smaller subset of antibodies that have utility in cell-based IF from a larger pool of hits.
- HCI can also be used during antibody development to validate antibodies for use in cell-based IF.
- The process has enabled the discovery and validation of hundreds of highly specific antibodies with utility in cell-based IF.
- CST leverages expertise in antibody conjugation to provide directly conjugated antibodies for use in HCI, reducing the staining workflow required for immunofluorescence protocols.
- When selecting antibodies for your HCI experiments from CST, look for antibodies marked with Immunofluorescence (IF) validation.

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