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Assay Optimization on the U-PLEX® Assay Platform

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1 Abstract

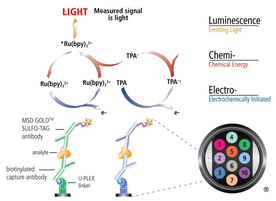
Purpose: The U-PLEX assay platform is designed to enable flexible creation of multiplex immunoassays using MSD's MULTI-ARRAY® technology. Throughout the development process, we streamlined the tools and protocols to provide a platform that is amenable to rapid assay development using a wide selection of binding reagents and antibody pairs.

Methods: The U-PLEX platform consists of 10 unique U-PLEX linkers that specifically bind to 10 individual locations ("spots") at the bottom of a 96-well U-PLEX plate. Each linker has a biotin-binding domain that couples to a biotinylated capture reagent as well as a domain that binds to its corresponding spot on the plate. Reagent conjugations were conducted with Sulfo-NHS-LC-Biotin and MSD SULFO-TAG™. Critical reagents (antibody pairs, calibrators, diluents) were selected from existing MSD® assays in order to facilitate characterization of the assays on the U-PLEX platform. Each assay was characterized for a number of parameters to enable analysis of the overall optimization process.

Results: Biotin to protein challenges were evaluated at ratios of 5:1, 10:1, and 20:1, with optimal signals resulting from a 10:1 ratio. Capture antibody concentrations were optimal at levels above 0.3 µg/mL. Over 40 detection antibodies were evaluated in the range of 0.25–2.0 µg/mL and showed minimal non-specific binding to the U-PLEX plates across the range (typically less than 0.1% of maximum signal). Assay protocols were evaluated with capture antibody immobilization from 1 hour to overnight, analyte incubations from 1 to 8 hours, and detection antibody incubations from 0.5 to 2 hours. Upon optimization, assays demonstrated sensitivities in the pg/mL range. Over 40 analytes were readily multiplexed in any combination. Typically, assays developed on the U-PLEX platform showed performance characteristics comparable to other MSD platforms, with a 3–4 log dynamic range and a low sample volume requirement (typically 25 µL) to enable conservation of precious samples with no compromise in performance.

2 Methods

MSD's electrochemiluminescence detection technology uses SULFO-TAG labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY and MULTI-SPOT® microplates. The U-PLEX assay platform utilizes 10 unique linkers that specifically bind to individual spots enabling simple and flexible creation of multiplex immunoassays.



Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

Typical U-PLEX Protocol

Create U-PLEX Coating Solution:

- Dilute each biotinylated antibody to 10 µg/mL in coating diluent.
- Couple each biotinylated antibody to a unique linker by adding 200 µL of the antibody to 300 µL of the assigned linker. Vortex. Incubate at room temperature (RT) for 30 minutes.
- Add 200 µL of Stop Solution. Vortex. Incubate at RT for 30 minutes.
- Combine 600 µL of each U-PLEX-coupled antibody solution into a single tube and vortex. Up to 10 U-PLEX-coupled antibodies can be pooled.

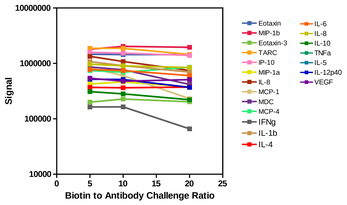
Coat U-PLEX Plate:

- Add 50 µL of multiplex coating solution to each well. Seal plate with an adhesive plate seal and incubate at RT with shaking for 1 hour or overnight.
- Wash plate 3 times with at least 150 µL/well of PBS-T or MSD Wash Buffer.

Complete (Run Assay):

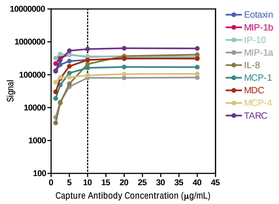
- Add 50 µL of sample, calibrator, or control to each well; shake for 1 hr and wash.
- Add 50 µL of detection antibody solution to each well; shake for 1 hr and wash.
- Add 150 µL of 2X Read Buffer T to each well and read.

3 Coupling U-PLEX Linkers: Biotin Challenge Ratio Optimization



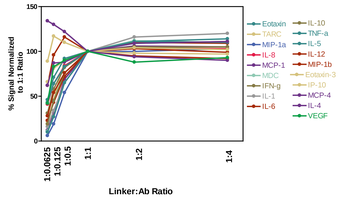
Twenty assays were chosen to optimize the biotin challenge ratio. Results showed that a challenge ratio of 5:1 or 10:1 is optimal for most assays. MSD recommends a 10:1 biotin challenge ratio for all U-PLEX assays. Over-conjugating antibodies with biotin can lead to decreased assay performance.

4 Coupling U-PLEX Linkers: Capture Antibody Titration



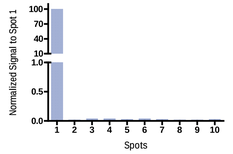
The antibody concentration coupled to a constant U-PLEX linker concentration was tested on over 30 different assays during development (data from 9 assays are presented above). MSD recommends using 10 µg/mL of capture antibody. At this concentration, less than 2 µg of antibody is used per 96-well plate. The capture antibody concentration could be reduced to conserve antibody.

5 Coupling U-PLEX Linkers: Effects of Changing the Linker to Antibody Ratio



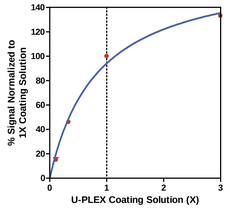
Twenty functional assays were tested at various linker to biotinylated Ab ratios. The recommended protocol of 300 µL linker with 200 µL of biotin-Ab (10 µg/mL) results in a 1:1 molar ratio. Increasing the antibody ratio relative to the linker does not improve assay signals. The extent of signal suppression is assay dependent. The majority of the assays will lose 25-50% of signals if the antibody ratio is reduced by half.

6 Coupling U-PLEX Linkers: Effectiveness of the Stop Solution



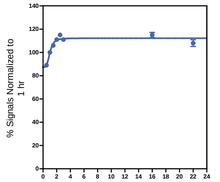
The Stop Solution is effective in preventing the binding of unbound antibodies and linkers. All spots with unbound linkers (spots 2-10) showed insignificant signals that were less than 0.04% of the spot with linker-coupled antibody (spot 1). The same test was repeated on all 10 linkers. The results indicate that the Stop Solution is effective for all the linkers (data not shown).

7 Coating U-PLEX Plate: Titration of Coating Solution



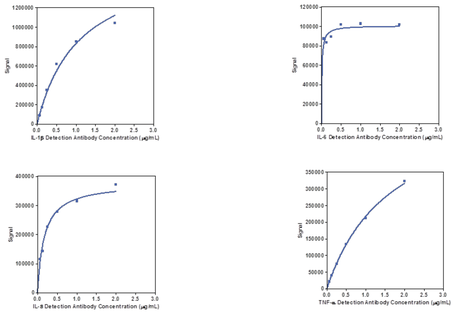
The U-PLEX coating solution was optimized for the capacity of the plates. The plates have a capacity of approximately 0.1 pmoles. Increasing the concentration of the U-PLEX coating solution by 3-fold resulted in a 30% increase in signal. Decreasing the concentration by 3-fold reduced the signal by approximately 50%.

8 Coating U-PLEX Plate: Optimization of Incubation Time



The U-PLEX coating solution was incubated on the plates at room temperature with shaking. The incubation times ranged from 30 min to 22 hours. Signals were normalized to the 1 hour coating time. Plates can easily be coated at room temperature or 4°C overnight, or at room temperature for 1 hour and then stored at 4°C overnight if needed. Plate coating time can be reduced to ½ hour if necessary for feasibility or screening studies.

9 Antibody Concentration Optimization: Detection Antibody Titration



Titration of the detection antibody should be done for each specific reagent to determine the best detection antibody concentration. Typical range is from 0.1 µg/mL to 3 µg/mL. For most high affinity antibodies, the optimal detection antibody concentration will be less than 0.5 µg/mL. When developing a multiplexed assay, the best practice is to test detection antibodies individually to determine the impact of the detection antibody concentration on the background signals for each of the capture antibodies.

10 Non-specific Binding of Functional Assay

Analytes	Eotaxin	Eotaxin-3	IP-10	MCP-1	MCP-4	MDC	MIP-1α	MIP-1β	TARC
Eotaxin	100.00%	0.16%	0.01%	0.00%	0.03%	0.01%	0.01%	0.01%	0.01%
Eotaxin-3	-0.01%	100.00%	0.05%	0.01%	0.07%	0.25%	0.04%	0.00%	0.09%
IP-10	0.01%	0.05%	100.00%	0.00%	0.02%	0.12%	0.01%	0.01%	0.02%
MCP-1	0.01%	0.03%	0.01%	100.00%	0.12%	0.01%	0.01%	0.07%	0.04%
MCP-4	0.00%	0.01%	0.00%	0.00%	100.00%	0.01%	0.00%	0.00%	0.01%
MDC	0.02%	0.04%	0.03%	0.02%	0.01%	100.00%	0.02%	0.01%	0.02%
MIP-1α	0.02%	0.05%	0.13%	0.02%	0.06%	0.41%	100.00%	0.04%	0.05%
MIP-1β	0.01%	0.04%	0.01%	0.01%	0.06%	0.02%	0.05%	100.00%	0.04%
TARC	0.01%	0.03%	0.01%	0.00%	0.01%	0.01%	0.01%	0.00%	100.00%

To assess non-specific binding, 9 assays were tested individually (single calibrator and single detection). Non-specific binding was less than 0.5% for all the assays. Additional non-specific binding data can be found on a related poster (NBC-15-487).

11 Conclusions

- The U-PLEX platform was optimized over a variety of conditions for coupling U-PLEX linkers and coating U-PLEX plates.
- Choosing the appropriate detection antibody concentration is a critical step in assay optimization. In addition, antibody selection, conjugation, and optimization of calibration curve are other critical parameters that need to be considered in assay development.
- Overall, it was demonstrated that numerous assays can be readily developed and optimized on the U-PLEX platform. The ease of use and flexibility of this platform uniquely enables the creation of custom multiplex panels with robust performance. Thus, the user can quickly construct multiplexed panels of analytes optimized for sensitivity, specificity, and workflow.

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