Luminex

# Dual Reporter Functionality of the xMAP INTELLIFLEX® DR-SE System

**This Technical Note describes several experiments that were conducted to evaluate the dual reporter function of the xMAP INTELLIFLEX® DR-SE System. Detailed protocols, the materials used, and representative results are shown below.**

## Dual Reporter Oligonucleotide Hybridization on the Luminex xMAP INTELLIFLEX DR-SE System – 2-Plex Assay Example

In this study, we evaluated the dual reporter functionality of the xMAP® INTELLIFLEX DR-SE System using an xTAG® oligonucleotide (oligo) hybridization assay. We evaluated different pairs of reporter dyes in various hybridization buffers to determine if two dyes that bind to different oligo targets on different bead sets can be used in a single reaction without interference. Historically, xMAP® platforms use a single fluorescent reporter channel (RP1) to measure and quantify the amount of a specific analyte bound to a microsphere. The green laser excites the reporter fluorochrome bound to the microsphere surface at 532 nm and measures the emitted orange fluorescence at 565–585 nm. The new xMAP INTELLIFLEX DR-SE System has a second reporter channel (RP2) that uses an additional violet laser. The violet laser excites the reporter fluorochrome bound to the microsphere surface at 405 nm and measures the emitted blue fluorescence at 421–441 nm.

The **MagPlex®-TAG™** bead regions used for this study were region 14 and region 22. The reporter dye combinations assessed were Alexa Fluor™ 532 (A532) with Alexa Fluor 405 (A405) and A532 with Streptavidin Super Bright 436 (SASB). All reactions were evaluated using two different hybridization buffers – 1X xTAG® hybridization buffer and 1X TMAC hybridization buffer. TAG-14 and TAG-22 **target oligos** pre-labeled at the 5' end with A532, A405, or biotin were used in this study. The assay protocol is available in the xMAP Cookbook, 6th edition, Protocol 5.3.4.

### Reagents Required:

| Reagents and Consumables | Vendors |
|---|---|
| DNA oligos | Integrated DNA Technologies, Inc. |
| MagPlex®-TAG™ microspheres | Luminex (Regions 14 and 22)<br>MTAG-A014<br>MTAG-A022 |
| 1X xTAG® (Tm) hybridization buffer<br>(0.1 M Tris, pH 8.0, 0.2 M NaCl, 0.08% Triton™ X-100) | MilliporeSigma T3038<br>MilliporeSigma S5150<br>MilliporeSigma T8787 |
| 1X TMAC hybridization buffer<br>(3M TMAC, 0.1% Sarkosyl solution, 50 mM Tris-HCL, 4 mM EDTA, pH 8.0) | Sigma T3411<br>Sigma L7414<br>Sigma T3038<br>Thermo Fisher 15575020 |
| Streptavidin Super Bright 436 conjugate | Thermo Fisher 62-4317-82 |
| Bovine serum albumin solution | MilliporeSigma B8667-5ML |
| 96-well PCR plate | Thermo Fisher AB0600 |
| Thermocycler with 96-well head and heated lid | Any suitable brand |
| Microseal® 'A' film | BioRad MSA5001 |
| 1.5 mL microcentrifuge tubes | USA Scientific 1415-2500, Eppendorf Protein LoBind®, 022431081 or equivalent |
| Disposable pipette tips | Any suitable brand |
| Distilled deionized water ($ddH_2O$) | Any suitable brand |

### Protocol [Dual Reporter Hybridization to MagPlex-TAG™ Microspheres on the xMAP INTELLIFLEX® DR-SE, 6th edition, Protocol 5.3.4]:

1. Select the appropriate MagPlex®-TAG™ microsphere sets and mix by vortex and sonication for approximately 20 seconds.
2. Resuspend in 1X xTAG® hybridization buffer or 1X TMAC hybridization buffer at a concentration of 2,500 microspheres of each set per 25 µL (100 microspheres/µL). Mix by vortex.
3. Aliquot 25 µL of the MagPlex-TAG microsphere mixture to each well (2,500 beads of each set/reaction).
4. Add 25 µL of the appropriate hybridization buffer to each background well.
5. Dilute the oligos in 1X xTAG hybridization buffer or 1X TMAC hybridization buffer to obtain the appropriate amount (fmols) in 25 µL.
6. Add 25 µL of each oligo sample to the appropriate wells. Mix gently by pipette.
7. Cover the plate with Microseal 'A' film to prevent evaporation and hybridize in a thermal cycler with the following parameters:
    a. 96°C for 90 seconds
    b. 37°C for 15–30 minutes

    *Note: Set the second step to 37°C FOREVER and use a timer to monitor the 15–30 minute hybridization time.*
8. Prepare reporter mix (if needed) by diluting the SASB reporter to 1.3 µg/mL in 1X xTAG or 1X TMAC hybridization buffer containing 0.2% BSA.
9. Add 50 µL of reporter mix or hybridization buffer to the appropriate wells of the plate in the thermal cycler (total volume 100 µL/well). Mix gently by pipette.
10. Incubate at 37°C for 10–15 minutes in the thermal cycler with heated lid.
11. Analyze 75 µL at 37°C on the xMAP® INTELLIFLEX DR-SE analyzer according to the system manual.

## Results and Conclusions:

In this study, we evaluated different reporter dye combinations on different target oligos that bind to different bead regions in a no wash hybridization assay using 1X xTAG and 1X TMAC hybridization buffers. The dye pairs tested were – a) A532 (RP1) with A405 (RP2) and b) A532 (RP1) with SASB (RP2). The median fluorescence intensity (MFI) was comparable for each reporter dye, whether it was used separately (single reporter) or in combination for the dual reporter assay. Data are shown for A532 paired with TAG-14 and A405 or SASB paired with TAG-22 **(Figures 1–3)**. The results were similar when the alternate TAG oligo was used with each dye, i.e., A532 with TAG-22 and A405 with TAG-14 (data not shown).

No impact or interference on signal was observed when both reporter dyes were used in the same reaction in 1X xTAG hybridization buffer **(Figures 1 and 3)**. Interestingly, when using 1X TMAC hybridization buffer, the signals for both A532 and A405 were enhanced **(Figure 2)**. The signal for A405 was approximately 20–30% less than the A532 signal, regardless of the hybridization buffer used. In this study, the final concentration of SASB in the reaction was 0.625 µg/mL, which performed adequately in 1X xTAG hybridization buffer, but not in 1X TMAC hybridization buffer. In 1X TMAC, SASB showed high backgrounds and non-specific signals, presumably due to non-specific binding of the dye to both bead sets in all reactions (data not shown). Further reduction of the SASB concentration did not alleviate the high background signals.

Overall, the data show that the dual reporter function of the xMAP INTELLIFLEX DR-SE System can be used successfully for detecting different oligonucleotide targets captured onto different bead regions without any interference between the different dyes and the reporter channels.

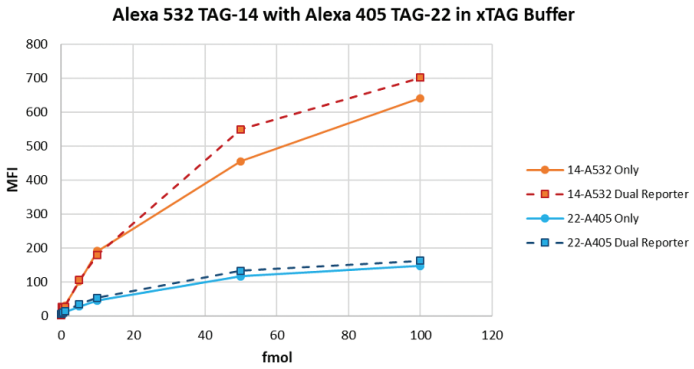


**Figure 1:** The A532-labeled TAG-14 oligo target was evaluated with A405-labeled TAG-22 oligo target in 1X xTAG® hybridization buffer. A532 = Alexa Fluor™ 532, A405 = Alexa Fluor™ 405.

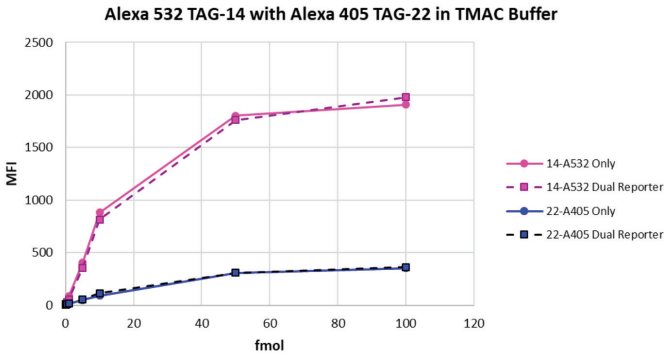


**Figure 2:** The A532-labeled TAG-14 oligo target was evaluated with A405-labeled TAG-22 target in 1X TMAC hybridization buffer. A532 = Alexa Fluor™ 532, A405 = Alexa Fluor™ 405.

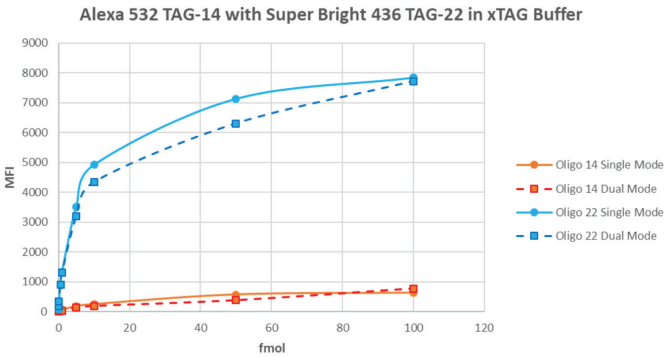


**Figure 3:** The A532-labeled TAG-14 oligo target was evaluated with biotinylated TAG-22 oligo target + SASB in 1X xTAG® hybridization buffer. A532 = Alexa Fluor™ 532, SASB = Streptavidin Super Bright 436.

# Dual Reporter Antibody Isotyping on the Luminex xMAP® INTELLIFLEX DR-SE System

Antibody isotyping assays are used to measure the classes and/or subclasses of antibodies in a serum sample and can be used to monitor the immune response in general or to a specific infection, vaccination, or drug therapy. In this study, we evaluated the dual reporter functionality of the xMAP® INTELLIFLEX DR-SE System in an antibody isotyping assay for SARS-CoV-2 with various pairs of reporter dyes. Historically, xMAP® platforms use a single fluorescent reporter channel (RP1) to measure and quantify the amount of a specific analyte bound to a microsphere. The green laser excites the reporter fluorochrome bound to the microsphere surface at 532 nm and measures the emitted orange fluorescence at 565–585 nm. The new xMAP INTELLIFLEX DR-SE System has a second reporter channel (RP2) that uses an additional violet laser. The violet laser excites the reporter fluorochrome bound to the microsphere surface at 405 nm and measures the emitted blue fluorescence at 421–441 nm.

In this study, we used the Luminex **xMAP® SARS-CoV-2 Multi-Antigen IgG Assay** (Multi-Ag) with serum samples previously tested for IgG using R-phycoerythin (R-PE). We adapted the Multi-Ag assay protocol [based on the indirect (serological) immunoassay method], and modified it for a dual reporter isotyping assay [xMAP® Cookbook, 6th Edition, Protocol 4.3.3.2]. The reporter dye combinations assessed in this study were R-PE with Brilliant Violet™ (BV) and R-PE with Streptavidin Super Bright 436 (SASB). The R-PE and the SASB dyes were used to measure both IgG and IgM, whereas the BV dye was used to measure IgG only.

## Reagents Required:

| Reagents and Consumables | Vendors |
|---|---|
| Serum samples | Any suitable source |
| MagPlex® microspheres | Obtained from Luminex xMAP® SARS-CoV-2 Multi-Antigen IgG Assay, 30-00124 |
| PBS–TBN Buffer*<br>(PBS + 0.1% BSA + 0.02% Tween-20 + 0.05% sodium azide) | MilliporeSigma P3813<br>MilliporeSigma A7888<br>MilliporeSigma P9416<br>MilliporeSigma S8032 |
| PE anti-IgM detection antibody | Jackson ImmunoResearch Laboratories 109-116-129 |
| PE anti-IgG detection antibody | Jackson ImmunoResearch Laboratories 109-116-088 |
| Biotinylated anti-IgG detection antibody | Jackson ImmunoResearch Laboratories 109-065-003 |
| Biotinylated anti-IgM detection antibody | Jackson ImmunoResearch Laboratories 109-065-043 |
| BV anti-IgG detection antibody | Jackson ImmunoResearch Laboratories 709-675-149 |
| Streptavidin Super Bright 436 conjugate | Thermo Fisher 62-4317-82 |
| 96-well, round-bottom polystyrene solid plates | Corning® 3789A |
| 96-well plate magnet | PerkinElmer® |
| Plate shaker | Any suitable brand |
| 1.5 mL microcentrifuge tubes | USA Scientific®, 1415-2500, Eppendorf Protein LoBind®, 022431081, or equivalent |
| Disposable pipette tips | Any suitable brand |

**Used both as assay buffer and microsphere wash buffer.*

## Protocol [Adapted from xMAP® SARS-CoV-2 Multi-Antigen IgG Assay Protocol, see xMAP® Cookbook, 6th Edition, Protocol 4.3.3.2]

1. Dilute the serum samples to appropriate concentrations (e.g., 1:400) using PBS-TBN buffer.
2. Add 50 µL of the diluted samples to the appropriate wells.
3. Select the appropriate antigen-coupled microsphere sets. Prepare a working microsphere mixture by diluting the coupled microsphere stocks to a final concentration of 50 microspheres of each set/µL in PBS-TBN buffer (~2,000 beads/set).
4. Resuspend the microspheres by vortex for approximately 30 seconds.
5. Aliquot 50 µL of the working microsphere mixture to the appropriate wells.
6. Cover the plate to protect it from light and incubate for 60 minutes at room temperature on a plate shaker set to approximately 800 rpm.
7. Place the plate onto the magnetic separator and allow separation to occur for 120 seconds.
8. With the plate still on the magnetic separator, carefully remove the supernatant from each well either by manual inversion, manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
9. Remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well. Mix gently using a pipette.
10. Repeat steps 7–8 once more for a total of two washes and proceed to step 11.
11. Remove the plate from the magnetic separator and add 100 µL of appropriate detection antibody/antibodies (at 1 µg/mL) to each well.
12. Cover the plate to protect it from light and incubate for 60 minutes at room temperature on a plate shaker set to approximately 800 rpm.
13. Place the plate onto the magnetic separator and allow separation to occur for 120 seconds.
14. With the plate still on the magnetic separator, carefully remove the supernatant from each well either by manual inversion, manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
15. Remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well.
16. Repeat steps 13 and 14 once more for a total of two washes and proceed to either step 17 or 18.
17. **If using directly labeled detection antibody**, remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well. Resuspend the microspheres by pipetting up and down several times with a multichannel pipette or by placing the plate onto a plate shaker for approximately 15 seconds, then proceed to step 19.
18. **If using biotinylated detection antibody for one of the reporter channels**, perform the following steps:
    a. Remove the plate from the magnetic separator and add 100 µL of reporter conjugate to each well of the plate.
    *Note: The final concentration/well was 0.5–1.0 µg/mL for SASB in this study.*
    b. Cover the plate to protect it from light and incubate for 20 minutes at room temperature on a plate shaker set to approximately 800 rpm.
    c. Place the plate onto the magnetic separator and allow separation to occur for 120 seconds.
    d. With the plate still on the magnetic separator, carefully remove the supernatant from each well either by manual inversion, manual pipetting, or magnetic plate washer. Take care not to disturb the microspheres.
    e. Remove the plate from the magnetic separator and add 100 µL of wash buffer to each reaction well.
    f. Repeat steps 18c–18e once more for a total of two washes.
    g. Resuspend the microspheres by pipetting up and down several times with a multichannel pipette or placing the plate onto a plate shaker for approximately 15 seconds. Proceed to step 19.
19. Analyze 75 µL on the xMAP® INTELLIFLEX DR-SE System according to the system manual.

## Results and Conclusions:

In this study, three different commercially available reporter dye/antibody pairs were tested on the xMAP INTELLIFLEX DR-SE:
a) SASB anti-IgM with PE anti-IgG, b) PE anti-IgM with SASB anti-IgG, and c) PE anti-IgM with BV anti-IgG. The median fluorescence intensity (MFI) was comparable for each detection antibody/reporter, whether it was used separately (single reporter) or in combination for the dual reporter assay. Similar results were observed for all three SARS-CoV-2 antigens (RBD, N, and S). Representative data for the N antigen is shown in **Figures 1-3.**

Using PE-labeled anti-IgG with SASB and biotinylated anti-IgM provided the highest IgM signal, but using PE-labeled anti-IgM with BV-labeled anti-IgG eliminated an additional labeling step. Overall, the data showed that the dual reporter function of the xMAP INTELLIFLEX DR-SE System can be successfully used for detecting different protein molecules (i.e., two antibody isotypes) captured onto the same bead without any interference between the different dyes and the reporter channels.

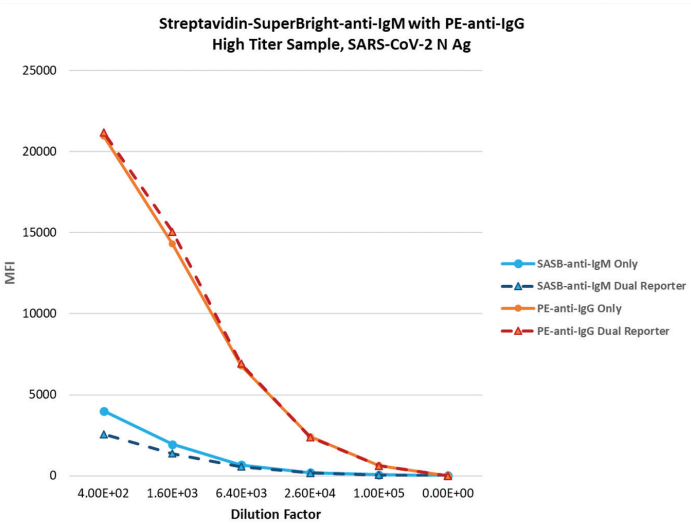


**Figure 1:** SASB-anti-IgM + R-PE-anti-IgG. Tested using a high titer sample for both IgG and IgM (SARS-CoV-2 N antigen). SASB = Streptavidin Super Bright 436 Conjugate, R-PE = R-phycoerythrin.

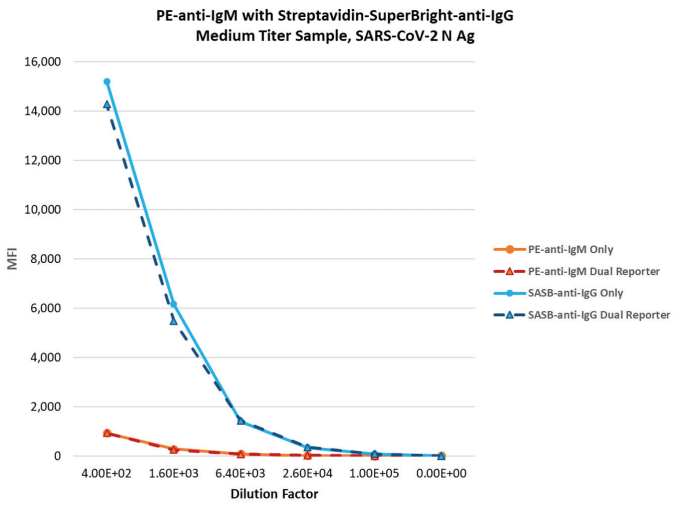


**Figure 2:** R-PE-anti-IgM + SASB-anti-IgG. Tested using a medium titer sample for both IgG and IgM (SARS-CoV-2 N antigen). SASB = Streptavidin Super Bright 436 Conjugate, R-PE = R-phycoerythrin.

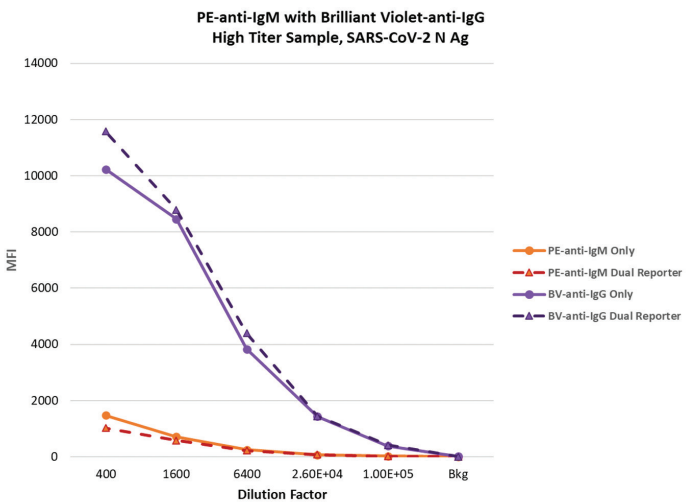


**Figure 3:** R-PE-anti-IgM + BV-anti-IgG. Tested using a high titer sample for both IgG and IgM (SARS-CoV-2 N antigen). BV = Brilliant Violet, R-PE = R-phycoerythrin.

# Dual Reporter Target Hybridization on the Luminex xMAP® INTELLIFLEX DR-SE System – Two Reporter Singleplex Assay Example

In this study, we evaluated the dual reporter functionality of the xMAP® INTELLIFLEX DR-SE System using a DNA hybridization assay with different pairs of reporter dyes to determine if two dyes that bind to the same target on the same bead set can be used in a single reaction without any interference or impact on reporter signals. Historically, xMAP® platforms use a single fluorescent reporter channel (RP1) to measure and quantify the amount of a specific analyte bound to a microsphere. The green laser excites the reporter fluorochrome bound to the microsphere surface at 532 nm and measures the emitted orange fluorescence at 565–585 nm. The new xMAP INTELLIFLEX DR-SE System has a second reporter channel (RP2) that uses an additional violet laser. The violet laser excites the reporter fluorochrome bound to the microsphere surface at 405 nm and measures the emitted blue fluorescence at 421–441 nm.

The MagPlex®-TAG™ bead region used for this study was region 44 and the reporter dye combination assessed was Alexa Fluor™ 532 (A532) with Streptavidin Super Bright 436 (SASB). The target used was a custom 55-nucleotide oligonucleotide (oligo) comprised of the 5' TAG-44 sequence with sequence from the bacteriophage MS2 genome. Oligo targets were pre-labeled with A532 at the 5' or 3' end, biotin at the 5' or 3' end, and dual-labeled with either 5' A532 + 3' biotin or 5' biotin + 3' A532. The assay protocol The assay protocol is available in the xMAP Cookbook, 6th edition, Protocol 5.3.4.

**Reagents Required:**

| Reagents and Consumables | Vendors |
|---|---|
| DNA oligos | Integrated DNA Technologies, Inc. |
| MagPlex®-TAG™ microspheres | Luminex (Region 44), MTAG-A044 |
| 1X xTAG® hybridization buffer<br>(0.1 M Tris, pH 8.0, 0.2 M<br>NaCl, 0.08% Triton® X-100) | MilliporeSigma T3038<br>MilliporeSigma S5150<br>MilliporeSigma T8787 |
| Streptavidin Super Bright 436 conjugate | Thermo Fisher 62-4317-82 |
| Bovine serum albumin solution | MilliporeSigma B8667-5ML |
| 96-well PCR plate | Thermo Fisher AB0600 |
| Thermocycler with 96-well head and heated lid | Any suitable brand |
| Microseal® 'A' film | Bio-Rad MSA5001 |
| 1.5 mL microcentrifuge tubes | *USA Scientific 1415-2500*<br>*Eppendorf Protein LoBind®, 022431081 or equivalent* |
| Disposable pipette tips | *Any suitable brand* |
| Distilled deionized water ($ddH_2O$) | *Any suitable brand* |

### Protocol [xMAP® Cookbook, 6th Edition, Protocol 5.3.4]:

1. Select the appropriate MagPlex®-TAG™ microsphere set and mix by vortex and sonication for approximately 20 seconds.
2. Resuspend in 1X xTAG® hybridization buffer at a concentration of 2,500 microspheres of each set per 25 µL. Mix by vortex.
3. Aliquot 25 µL of the MagPlex-TAG microsphere mixture to each well (2,500 beads of each set/reaction).
4. Add 25 µL of the hybridization buffer to each background well.
5. Dilute the target oligos in 1X xTAG hybridization buffer to obtain the appropriate amount (fmols) in 25 µL.
6. Add 25 µL each oligo sample to the appropriate wells. Mix gently by pipette.
7. Cover the plate with Microseal 'A' film to prevent evaporation and hybridize in a thermal cycler with the following parameters:
    a. 96°C for 90 seconds
    b. 37°C for 15–30 minutes
    *Note: Set the second step to 37°C FOREVER and use a timer to monitor the 15–30 minute hybridization time.*
8. Prepare reporter mix by diluting reporter (SASB) to 1.3 µg/mL in 1X xTAG hybridization buffer containing 0.2% BSA.
9. Add 50 µL of reporter mix or hybridization buffer to the appropriate wells of the plate in the thermal cycler (total volume 100 µL/well). Mix gently by pipette.
10. Incubate at 37°C for 10–15 minutes in the thermal cycler with heated lid.
11. Analyze 75 µL at 37°C on the xMAP INTELLIFLEX DR-SE analyzer according to the system manual.

## Results and Conclusions:

In this study, we evaluated different reporter dye pairs bound to the same target molecule on a single bead region using a 55 nucleotide oligo target in a no wash hybridization assay with 1X xTAG hybridization buffer. The dye combination tested was A532 (RP1) with SASB (RP2). The median fluorescence intensity (MFI) was comparable for each reporter dye, whether it was used separately (single reporter) or in combination for the dual reporter assay. There was no effect or interference on signal when both dyes were bound to the same molecule on the same bead **(Figures 1 and 2).** Overall, the data demonstrated that the dual reporter functionality of the xMAP INTELLIFLEX DR-SE System can be successfully used for detecting a dual-labeled target molecule captured onto a single bead region without any interference between the different dyes and the reporter channels.

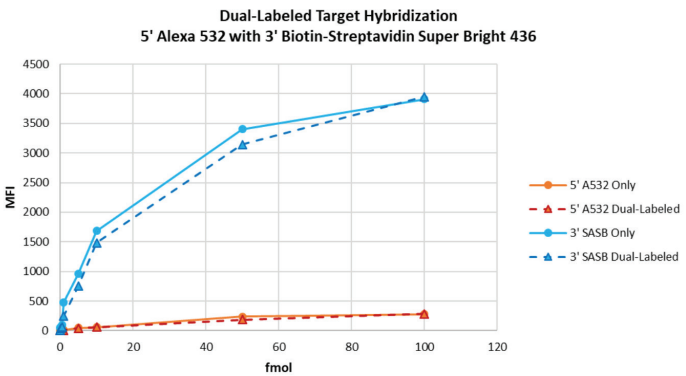


**Figure 1:** Dual-labeled target hybridization with A532 at the 5' end and SASB at the 3' end. A532 = Alexa Fluor™ 532, SASB = Streptavidin Super Bright 436.

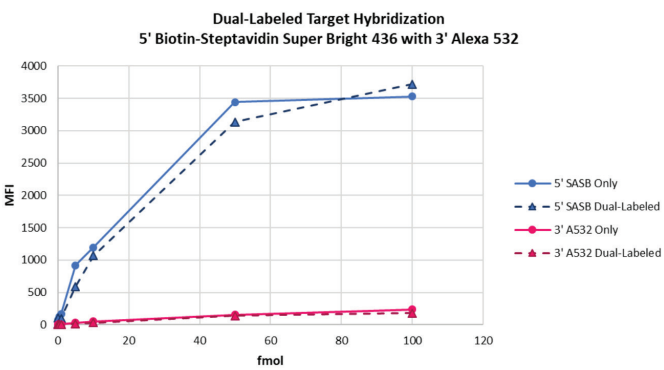


**Figure 2:** Dual-labeled target hybridization with SASB at the 5' end and A532 at the 3' end. A532 = Alexa Fluor™ 532, SASB = Streptavidin Super Bright 436.


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