Rapid, consistent, and flexible dispensing of AlphaLISA and HTRF reagents using the FlexDrop Plus non-contact dispenser.

Introduction

The FlexDrop™ Plus non-contact dispenser can dispense volumes as low as 8 nL into 96-, 384- or 1536-well plates, featuring 1 µL dead volumes with greater intra-assay precision than hand-pipetting. The FlexDrop Plus utilizes positive pressure coupled with light-based droplet detection to generate 8 to 50 nL droplets at a rate of 100 droplets per second. Common uses include dispensing compounds, biological samples, immunoassay reagents (HTRF® and AlphaLISA® reagents), and NGS or q-PCR preparation. The intraassay precision when dispensing AlphaLISA reagents using the FlexDrop Plus dispenser compared with pipetting by hand was examined, as well as the effect of dispensing AlphaLISA and HTRF reagents with droplet detection enabled on the resulting signal is presented in this tech note.

Materials and methods

AlphaLISA

AlphaLISA technology is a bead-based, no-wash immunoassay where analyte-specific antibodies are conjugated to Donor and Acceptor beads. In the presence of an analyte, the Donor and Acceptor beads come into proximity. Upon excitation at 680 nm, Donor beads release singlet oxygen that diffuses (up to 200 nm) into the analyte matrix and interacts with Acceptor beads, facilitating energy transfer events. This results in an emission maximum at 615 nm, which can be read on an Alpha-enabled plate reader.

A schematic illustrating the differences between AlphaScreen™ and AlphaLISA technologies is shown in Figure 1. The compatibility between the FlexDrop Plus and Alpha technology was initially assessed by dispensing AlphaScreen (Omnibeads #2266483 & TruHits #6760627) reagents. Because the FlexDrop Plus droplet detection uses light to measure the size of the droplets it is important to determine the effect of dispensing AlphaLISA, and HTRF reagents with droplet detection turned "on" and "off". The FlexDrop Plus dispensing precision was also evaluated and compared to pipetting reagents by hand.

To set up dispensing runs using AlphaScreen, AlphaLISA, and HTRF kit reagents, "Liquid Class" files were created in the FlexDrop Plus instrument software using buffers specific to each immuno-assay. The resulting files were stored in the FlexDrop Plus "Liquid Class Library". The "Liquid Class Library" contains the dispensing parameters needed to precisely dispense liquids with different viscosities, like aqueous liquids, DMSO, glycerol, and other solvents. Once the liquid class for each AlphaScreen assay (Omnibeads and TruHits kit) was defined in the FlexDrop Plus software, "Droplet verification" was disabled under the settings menu and the reagents were dispensed into replicate wells in the recommended destination/384-well assay OptiPlates (Revvity #6057480). The resulting Alpha signal was read on an Alpha-capable EnVision® 2105 multimode plate reader using pre-programmed, assay-specific settings. The summarized results from dispensing Omnibeads and TruHits beads are provided in Appendix 1.

HTRF

Homogenous Time-Resolved Fluorescence (HTRF) is a fluorescence resonance energy transfer (FRET) based technology. For a sandwich assay, two antibodies that recognize a protein of interest are used, with one antibody coupled to a donor, and the other with the acceptor. When donor and acceptor antibodies are bound to the analyte and exposed to a laser or flash lamp light source, FRET occurs between the donor and acceptor fluorophores, generating a signal proportional to the formation of analyte-antibody complexes. Illustrations of the HTRF assay kit protocols used in this tech note are provided in Figure 3.

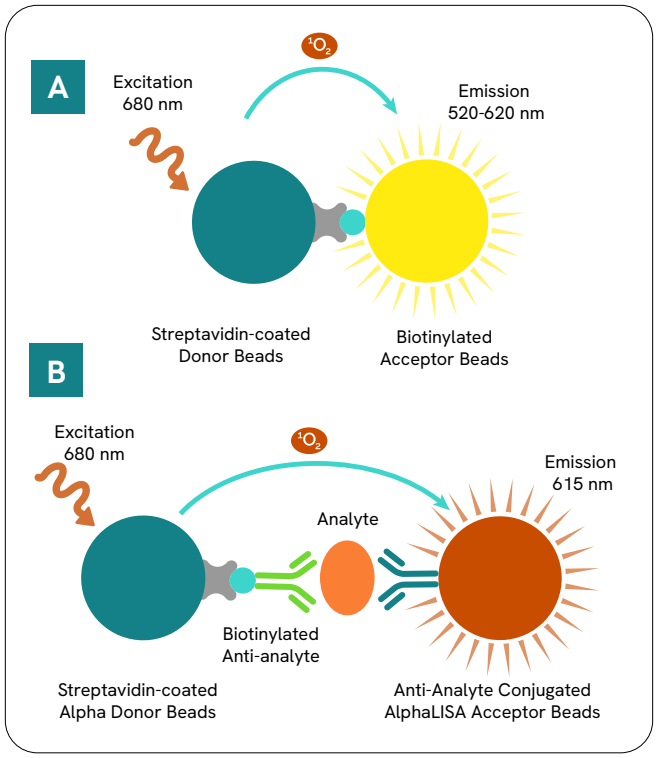

Figure 1: Bead chemistry for TruHits Kit AlphaScreen technology and AlphaLISA (general) immunoassay. A. TruHits Kit (AlphaScreen) contains Streptavidin-coated Donor beads and biotinylated Acceptor beads, forming a complex when combined in solution. B. AlphaLISA assays use antigen-specific antibodies to bring the conjugated Donor and Acceptor beads into proximity. Excitation of Donor beads at 680 nm results in emission from nearby Acceptor beads (520 – 620 nm for TruHits Acceptor beads and 615 nm for AlphaLISA Acceptor beads).

Results & discussion

AlphaScreen technology (Omnibeads & TruHits kit) is compatible with the FlexDrop Plus

The FlexDrop Plus 80 µL Source Well Plate (Revvity #CLS156444) loading parameters were the same for each experimental replicate. The corresponding number of wells analyzed from the destination plate, signal (counts), and related statistical information is listed in Appendix 1. Omnibeads™ have been designed as a tool to identify instrument-related variability in AlphaScreen assays. These beads contain all the chemical components necessary to generate a strong AlphaScreen signal without requiring the presence of both Acceptor and Donor beads. The TruHits kit is designed as a tool for AlphaScreen users to identify false positives in AlphaScreen HTS assays early in the screening process and in a cost-effective way. This kit includes Streptavidin Donor Beads and Biotinylated Acceptor Beads, which interact together to generate an AlphaScreen signal.

The AlphaScreen TruHits kit also identifies color quenchers, light scatterers (insoluble compounds), singlet oxygen quenchers, and biotin mimetics interfering with the AlphaScreen signal. Dispensing these reagents with the FlexDrop Plus droplet detection turned "off" allowed for the simultaneous assessment of feasibility and intra-assay precision, demonstrating that the FlexDrop Plus and AlphaLISA technology are compatible. Notably, the intra-assay CV for all replicates except for the Omnibeads Run 3 were < 5% when dispensing volumes ranging from 10 µL to 25 µL (Appendix 1). Differences in Max and Min Signal can be attributed to the use of different reagent aliquots.

Dispensing AlphaLISA reagents with the FlexDrop Plus dispenser can result in better intra-assay precision than pipetting by hand

Next, the FlexDrop Plus dispenser was used to dispense analyte standards and reagents from the AlphaLISA human TNFα Detection Kit (Revvity #AL208C) with droplet detection turned "off" and the results were directly compared with an AlphaLISA assay performed by hand-pipetting using a multi-channel repeat pipettor (125 µL – ThermoFisher Matrix™) into separate AlphaPlates (Revvity #6005350). The resulting scatter plot data is provided in Figure 2A.

The TNFα standard curves generated by dispensing with the FlexDrop Plus instrument and pipetting by hand deviated slightly at the top and bottom portions of the curve but largely overlapped in the linear range (Figure 2A). The signal intensity for the FlexDrop Plus instrument dispensed TNFα AlphaLISA standard curve was on average higher than pipetting by hand, resulting in an increased dynamic range. However, the LDL when pipetting by hand (1.8 pg/mL) was slightly lower than the FlexDrop Plus dispensed LDL (2.8 pg/mL). Interestingly, the CV calculated from the 12 background wells (containing no TNFα) was 2% lower when using the FlexDrop Plus instrument compared to manual pipetting (Appendix 2). This suggests that the FlexDrop Plus was slightly more precise in dispensing AlphaLISA reagents than pipetting by hand.

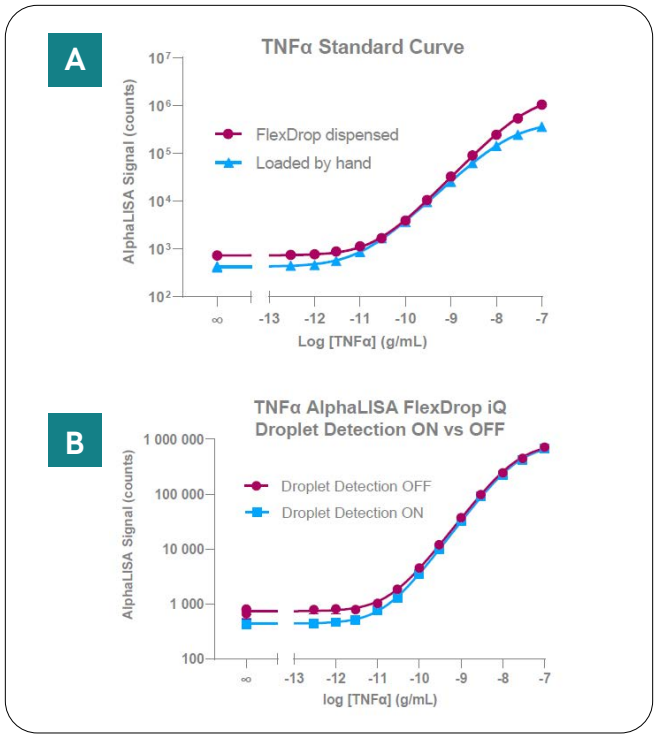

Figure 2: A. Superimposed standard curve from dispensing AlphaLISA human TNFα Detection Kit reagents with the FlexDrop Plus dispenservs. pipetting by hand. B. Superimposed standard curve from dispensing AlphaLISA human TNFα Detection Kit reagents with FlexDrop Plus dispenser with droplet detection turned "off" vs. turned "on".

Dispensing AlphaLISA reagents with Droplet Detection turned "on" can result in a reduction of signal

To determine the effect of droplet detection on the AlphaLISA signal, source wells were loaded with enough TNFα AlphaLISA reagents to dispense two complete standard curves with excess (once with droplet detection "off" and again with it turned "on"). In addition to running the standard curves, the TNFα standard was diluted to a concentration of 60,000 pg/mL and dispensed into 24 wells (12 with droplet detection turned "on" and 12 with it "off"). The FlexDrop Plus dispensing parameters for this work, resulting AlphaLISA signal, and corresponding statistical analysis are provided in Appendix 3. The effect of turning droplet detection "off" resulted in a slightly higher average signal than when turned "on" (Appendix 3A) with the highest deviation in signal at the bottom of the standard curve (Figure 2B). The intra-assay CV at each point on the standard curve was lower at TNFα concentrations above 100 pg/mL but significantly increased at concentrations below 100 pg/mL when droplet detection was turned "off" vs. "on" (Appendix 3A). Interestingly, the overall deviation in

calculated CV over the concentration range of the standard curve was lower when droplet detection was turned "on" (Appendix 3A). Taken together these results suggest that disabling droplet detection may impact intra-assay precision when dispensing samples with relatively low analyte concentrations. A TNFα concentration of 60,000 pg/mL was chosen to test the effect of turning droplet detection "on" while repeatedly dispensing the same concentration of analyte and AlphaLISA reagents across 12-wells in the destination plate using the FlexDrop Plus. This resulted in a gradual decrease in Alpha signal, resulting in an intra-assay CV of 13.4%. However, running the same dispensing parameters with droplet detection turned "off" resulted in an intra-assay CV of only 1.7% (Appendix 3B). Therefore, it is recommended that droplet detection be turned "off" when dispensing AlphaLISA reagents.

HTRF assays are unaffected by FlexDrop Plus droplet detection

The effect of droplet detection on HTRF assays was also determined using both the HTRF human TNFα Detection Kit (#62HTNFAPEG) and HTRF cAMP Gi Detection Kit (#62AM9PEB). The FlexDrop Plus instrument was used to dispense HTRF kit standards and reagents into the same HTRF plate (Revvity - 96-well low volume #66PL96025) with droplet detection turned both "on" and "off". The HTRF human TNFα Detection kit directly detects TNFα while the HTRF cAMP Gi Detection Kit measures the competitive binding of Eu cryptate-labeled cAMP and unlabeled cAMP with a d2-labeled antibody. Therefore, the HTRF signal decreases in proportion to the amount of unlabeled cAMP in a sample. Assay schematics for the HTRF kits are provided in Figure 3. The raw data and FlexDrop Plus dispensing parameters for the HTRF human TNFα Detection kit are shown in Appendix 4, and the respective data for the HTRF cAMP Gi Detection Kit can be found in Appendix 5.

The Eu cryptate and d2 anti-TNFα antibodies were pre-mixed prior to dispensing. TNFα standards were dispensed into the destination plate in triplicate. The HTRF ratio for human TNFα standards and reagents dispensed by the FlexDrop Plus was roughly the same when droplet detection was turned "on" or "off" (Figure 4A & Appendix 4A). The inter-assay CV calculated between droplet detection "on" and "off" runs at each point of the TNFα and cAMP standard curves were <3% and <8% respectively. This indicates that the droplet detection system of the FlexDrop Plus does not negatively affect the HTRF assay or corresponding signal.

The FlexDrop Plus instrument was used to dispense HTRF cAMP Gi Detection Kit analyte standards and reagents to determine if competition-assay-based HTRF assays are also unaffected by droplet detection. The analyte standard was diluted following the cAMP Gi Kit TDS and the FlexDrop Plus instrument dispensed the standards and reagents into triplicate wells in the destination plate (Appendix 5B). Again, the HTRF ratios were the same at each cAMP concentration when the kit reagents were dispensed with droplet detection turned "on" and "off" (Appendix 5A). The intra-assay CV at each cAMP concentration for both runs was <5% (except when 4.45 nM cAMP) when dispensed with droplet detection turned "off" (Appendix 5A). The resulting cAMP standard curves were again nearly identical (Figure 4B), reinforcing that droplet detection does not negatively affect the HTRF assay.

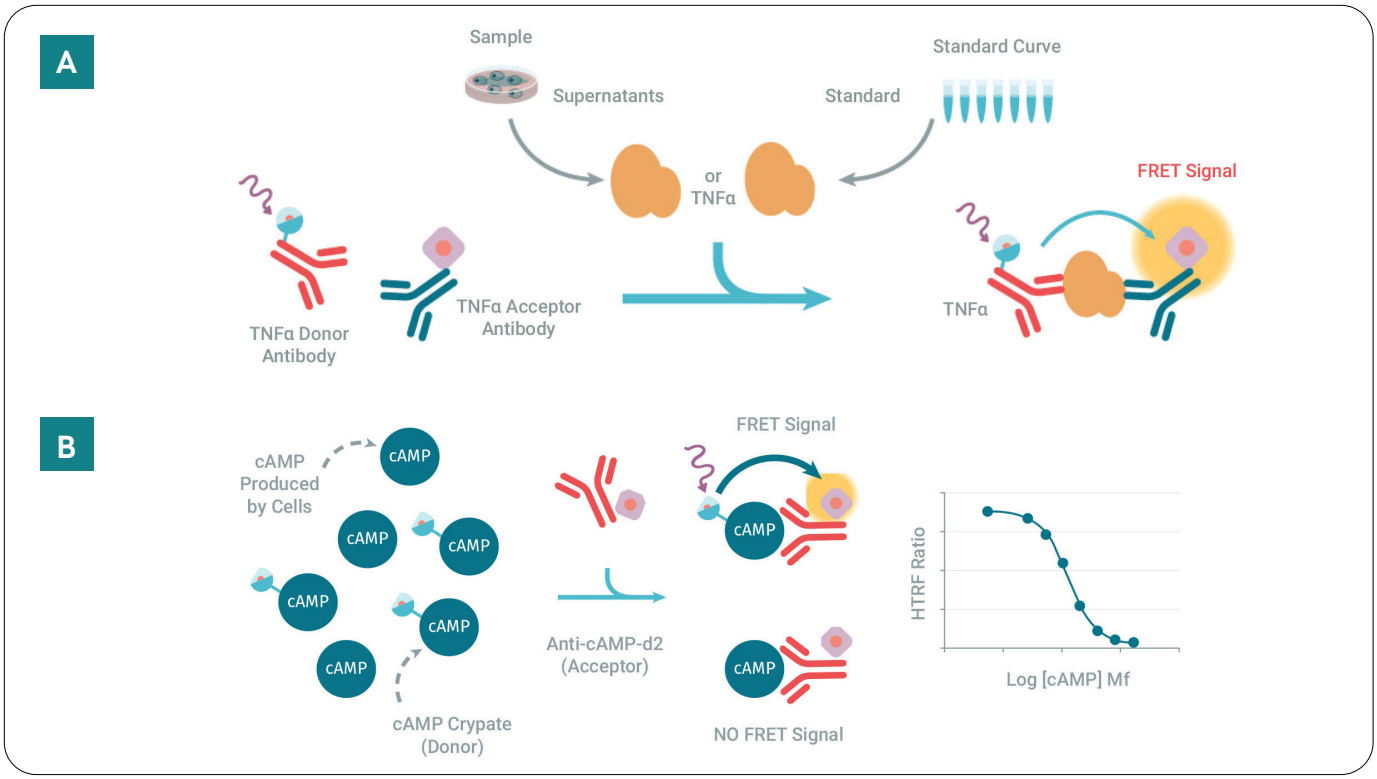

Figure 3: A.Assay schematic for HTRF human TNFα Detection Kit. B. Assay schematic for HTRF cAMP Gi Detection Kit (competition assay).

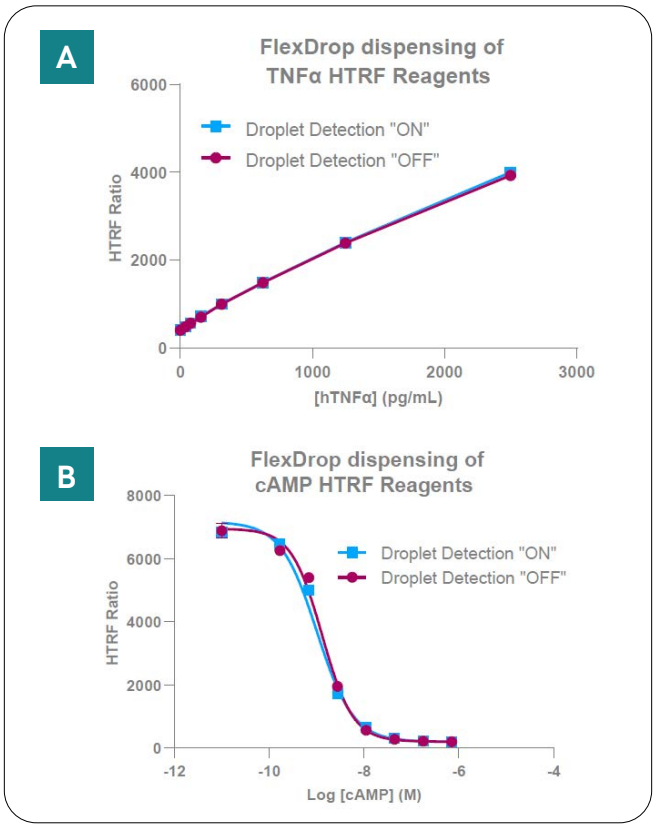

Figure 4: Superimposed standard curve calculated from the FlexDrop Plus dispensing of A. HTRF human TNFα Detection Kit and B. HTRF cAMP Gi Detection Kit standards and reagents with droplet detection turned "on" and "off"

Summary

This work has demonstrated that the FlexDrop Plus non-contact dispensing platform is compatible with AlphaScreen, AlphaLISA, and HTRF assay technologies. However, it is recommended that droplet detection be disabled when dispensing AlphaScreen and AlphaLISA reagents, to avoid the premature photoactivation of SA-Donor beads, which can lead to reduced signal and increased intra-assay CV (Appendix 3B). HTRF assays are unaffected by droplet detection (Figures 4A & 4B). Therefore, it is recommended to dispense HTRF reagents with droplet detection turned "on" for added precision and post-dispensing run analytics. Dispensing AlphaLISA reagents with the FlexDrop Plus can be more precise than pipetting by hand (Appendix 2A), however, the time to dispense reagents is much slower than hand pipetting (Appendix 3C, 4B & 5B). An added benefit to using the FlexDrop Plus dispenser is that the loading of source plate wells requires less reagent (an excess of a couple µL/well) than what is needed to fill reagent reservoirs for use with multichannel repeat-pipettes. This can significantly limit the amount of reagent wasted when setting up AlphaLISA or HTRF immunoassays. The FlexDrop Plus instrument would also be useful to dispense AlphaLISA or HTRF reagents when using repetitive workflows with numerous samples and complex loading schemes that may be susceptible to human error when pipetting by hand. For example, biomarker screening, high-throughput/high-content screening, or multiplex immunoassays.

Appendix

Appendix 1. Results for FlexDrop Plus dispensing of AlphaScreen reagents. All reagents were dispensed with droplet detection turned "off". Omnibeads (blue) and pre-mixed TruHits kit reagents (orange) were dispensed in 3 separate experimental runs. TruHits SA-Donor and b-Acceptor beads were loaded into separate source plate wells and dispensed individually into the same well in the destination plate (green) in 2 experimental runs. The max signal, min signal, average signal, standard deviation, and Coefficient of Variation CV = ((standard deviation / average signal) x 100%) were calculated for each experimental replicate.

Assay/Kit	Reagent	Source plate (CLS1546444)			384-well OptiPlate (Destination plate)						
		Volume loaded per well (µL)	# of wells	Volume dispensed per well (µL)	# of wells	Run #	MAX Signal (counts)	MIN Signal (counts)	Average Signal (counts)	Std. Dev.	Intra-assay CV
OmnibeadsKit	Omnibeads	70	72	25	190	1	223530	181408	207935.2	6477.9	3.1%
					192	2	239133	206968	225613.4	5251.5	2.3%
					190	3	193914	134306	178683.4	11537.7	6.5%
TruHits Kit (pre-mixed)	SA-Donor & b-Acceptor (mixed 1:1)	70	10	20	32	1	236091	211298	218313	5317.6	2.4%
						2	168826	145164	153601.3	5798.5	3.8%
						3	136581	124619	128716.4	2484.0	1.9%
TruHits Kit (reagents dispensed into same well)	SA-Donor beads	70	5	10	32	1	54954	45962	49681.4	2255.1	4.5%
	b-Acceptor beads										
	SA-Donor beads	70	5	10	32	2	133142	120476	124968.4	3529.2	2.8%
	b-Acceptor beads										

Appendix 2. FlexDrop Plus dispensing vs. hand-pipetting of AlphaLISA human TNFα Detection Kit reagents. The average signal, standard deviation, and intra-assay CV are listed for each [TNFα] on the standard curve. The TNFα kit standards were dispensed or pipetted into triplicate wells per TNFα concentration. Background wells contain no TNFα and were dispensed or pipetted into 12 wells.

	FlexDrop dispensed			Loaded by hand			
	[TNFα] (pg/ml)	Average Signal (counts)	Std. Dev.	CV (%)	Average Signal (counts)	Std. Dev.	CV (%)
TNFα Standard Dilutions	100000	1040537	6090	0.6%	358612	9789	2.7%
	30000	539358	28634	5.3%	244078	13898	5.7%
	10000	245430	6131	2.5%	143623	8079	5.6%
	3000	90424	1178	1.3%	62962	2902	4.6%
	1000	32561	838	2.6%	25481	217	0.9%
	300	10571	534	5.1%	9593	638	6.7%
	100	3914	145	3.7%	3684	87	2.4%
	30	1692	20	1.2%	1686	20	1.2%
	10	1137	59	5.2%	867	48	5.5%
	3	885	122	13.8%	566	20	3.5%
	1	772	61	7.9%	461	34	7.3%
	0.3	755	49	6.4%	447	17	3.8%
Background	N/A	726	37	5.1%	430	31	7.2%

Appendix 3. FlexDrop Plus dispensing of AlphaLISA human TNFα Detection Kit reagents with droplet detection "on" vs. "off". A. The average raw signal, standard deviation, and CV at each TNFα concentration with droplet detection turned "on" and "off". TNFα standards and AlphaLISA reagents dispensed by FlexDrop Plus. The "ON/OFF" Ratio was calculated by taking the average raw signal with droplet detection turned "on" divided by the signal with it turned "off". B. Results of dispensing 12 wells of 60,000 pg/mL TNFα with droplet detection "on" and 12 wells with it "off". C. Dispensing parameters related to both droplet detection "on" and "off" runs.

A

	FlexDrop dispensed			Loaded by hand				ON/OFF Ratio
	[TNFα] (pg/ml)	Average signal (counts)	Std. Dev.	CV	Average signal (counts)	Std. Dev.	CV	
TNFα Standard Dilutions	10000.0	716641	7372	1.0%	686488	5916	0.9%	0.96
	3000.0	449645	4535	1.0%	412439	8048	2.0%	0.92
	1000.0	245312	3164	1.3%	223739	8315	3.7%	0.91
	300.0	98123	528	0.5%	92242	3602	3.9%	0.94
	100.0	37286	457	1.2%	32575	1006	3.1%	0.87
	30.0	11978	295	2.5%	9991	634	6.3%	0.83
	10.0	4508	162	3.6%	3505	77	2.2%	0.78
	3.0	1852	186	10.1%	1315	53	4.0%	0.71
	1.0	1026	122	11.9%	761	17	2.3%	0.74
	3	791	113	14.3%	513	42	8.2%	0.65
	1	818	143	17.5%	466	14	3.1%	0.57
	0.3	793	137	17.3%	444	25	5.7%	0.56
Background	N/A	753	83	11.0%	443	45	10.2%	0.61

B

[TNFα] (pg/ml)	AlphaLISA signal (counts)	
	Droplet detection "OFF"	Droplet detection "ON"
60000	648100	611687
	634653	568096
	636304	578233
	633737	577965
	618271	545645
	635424	527321
	626405	456287
	620661	465498
	618226	479802
	620462	454534
	614484	408064
	613064	426928

Droplet detection "OFF"		
Average	St. Dev.	CV
626649	10840	1.7%

Droplet detection "ON"		
Average	St. Dev.	CV
508338	67873	13.4%

C Samples and dispensing parameters

- **Number of source plates used:** 1 - Droplet detection "on" and "off" dispensed from same source plate
- **Immunoassay buffer:** 50 µL/well x 4 source wells
  ↳ Dispensed: 5 µL/well x 12 wells
- **TNFα standards 1 - 12:** 50 µL/well x 1 source well per standard
  ↳ Dispensed: 5 µL/well x 3 wells per standard
- **6.00E-08 g/mL TNFα standard:** 75 µL/well x 2 source wells
  ↳ Dispensed: 5 µL/well x 12 wells
- **Acceptor beads & b-anti-TNFα antibody:** 75 µL/well x 38 source wells
  ↳ Dispensed: 20 µL/well x 60 wells
- **SA-Donor beads:** 75 µL/well x 52 source wells
  ↳ Dispensed: 25 µL/well x 60 wells

Time to dispense
6.5 minutes
6 minutes

Dispensing time for each run: **125 minutes**

Total dispensing time for droplet detection "ON" & "OFF" runs: **25 minutes**

Appendix 4. A. Data generated by dispensing human HTRF TNFα Detection Kit standards and reagents using the FlexDrop Plus with droplet detection turned "on" and "off". B. Source plate loading and dispensing parameters, and time to dispense

A	FlexDrop dispensed			Loaded by hand		
[TNFα] (pg/ml)	Average HTRF Ratio	Std. Dev.	CV (%)	Average HTRF Ratio	Std. Dev.	CV (%)
2500	3928.17	18.53	0.5%	3997.19	64.45	1.6%
1250	2388.75	6.40	0.3%	2404.00	18.19	0.8%
625	1486.74	11.81	0.8%	1490.52	21.15	1.4%
312.5	995.87	10.13	1.0%	997.08	25.07	2.5%
156.3	702.72	4.92	0.7%	716.30	13.14	1.8%
78.1	560.12	2.49	0.4%	568.39	4.83	0.9%
39.1	481.18	1.01	0.2%	484.14	6.23	1.3%
0	405.53	7.76	1.9%	406.30	2.87	0.7%

B Samples and dispensing parameters

- **Number of source plate(s) used:**
  1 (DD "on" & 1 DD "off" – same plate)
- **hTNFα standard 0 - 7:**
  70 µL/well x 2 source wells/standard
  ↳ Dispensed: 16 µL/ well x 3 wells/standard
- **Anti-hTNFα Eu cryptate & d2 antibody mix:** |
  70 µL/ well x 5 source wells
  ↳ Dispensed: 4 µL/well x 24 wells
- **Dispensing time per run - 2 minutes**
- **Dispensing time for both "ON" & "OFF" runs - 4 minutes**

Appendix 5. A. Data generated by the dispensing of HTRF cAMP Gi Detection Kit standards and reagents using the FlexDrop Plus with droplet detection turned "on" and "off". B. Source plate loading and dispensing parameters, and time to dispense.

A	Droplet Detection "OFF"			Droplet Detection "ON"		
[cAMP] (nM)	Average normalized signal	Std. Dev.	CV	Average normalized signal	Std. Dev.	CV
712	194.24	2.46	1.3%	195.64	3.29	1.7%
178	215.71	6.93	3.2%	213.11	4.10	1.9%
44.5	273.95	18.25	6.7%	302.25	7.20	2.4%
11.1	560.29	10.18	1.8%	643.67	5.88	0.9%
2.78	1952.08	28.54	1.5%	1728.47	13.98	0.8%
0.69	5393.89	41.78	0.8%	4993.37	138.67	2.8%
0.17	6259.19	6.43	0.1%	6456.46	28.18	0.4%
0	6883.44	235.96	3.4%	6845.09	24.24	0.4%
Positive Control	178.54	1.32	0.7%	177.01	2.71	1.5%

B Samples and dispensing parameters

- **Number of source plates used:**
  2 (1 - droplet detection "on", 1 - droplet detection "off")
- **Stimulation buffer:** 60 µL/well x 3 source wells.
  ↳ Dispensed: 5 µL/ well x 24 wells.
  10 µL/well x 3 wells (positive control).
- **cAMP standard 0 – 7: 25 µL/well x 1 source well.**
  ↳ Dispensed: 5 µL/ well x 3 wells/standard.
- **Eu cryptate:** 70 µL/well x 3 source wells.
  ↳ Dispensed: 5 µL/ well x 27 wells.
- **d2 antibody:** 60 µL/ well x 3 source wells.
  ↳ Dispensed: 5 µL/ well x 24 wells.
- **Lysis & Detection buffer:** 25 µL/ well x 1 source well.
  ↳ Dispensed: 5 µL/ well x 24 wells.
- **Dispensing Time per run - 1.5 minutes**
- **Dispensing Time for both "ON" & "OFF" runs - 3 minutes**

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
(800) 762-4000
www.revvity.com