TECHNICAL NOTE

AlphaLISA Ligand Binding Assays for Measurement of Human Immunoglobulin G Subclasses in Animal Sera

AlphaLISA #27

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AlphaLISA® Technology

These AlphaLISA immunodetection assays measure human IgG antibodies of subclasses 1 and 4 in monkey, mouse and rat sera.

Anti-Human IgG1 (AL141) and Anti-Human IgG4 (AL142) AlphaLISA Acceptor Beads

- AL141C/AL142C: 250 µg, 500 assay points*
- AL141M/AL142M: 5 mg, 10,000 assay points*
- AL141R/AL142R: 25 mg, 50,000 assay points*

*0.5 µg/assay point

Model System

Two human monoclonal antibodies against the pro-inflammatory cytokine human tumor necrosis factor alpha (hTNFα) were used to mimic biotherapeutic antibody drugs and show the capacity of AlphaLISA to measure various IgG subclasses in the sera of animals commonly used for pharmacokinetic studies. These anti-TNFα antibodies are fully human monoclonal antibodies and were generated by recombinant DNA technology. They feature the constant region of the human IgG1 isotype in one case or the constant region of the human IgG4 isotype in the other case. These two subclasses were chosen, because they are the most common types of IgGs used as biotherapeutic drugs.

AlphaLISA Assays

The AlphaLISA technology allows performing no-wash homogeneous proximity immunoassays using Alpha Donor and AlphaLISA Acceptor beads. In this technical note, we present the optimization of an assay for the detection of two subclasses of human immunoglobulin G (IgG1 and IgG4) in sera. Detection of IgGs was performed by the addition of anti-IgG1 or IgG4 antibodies in sera from different species, followed by the addition of biotinylated TNFα, Streptavidin (SA) Alpha Donor beads, and AlphaLISA Acceptor beads conjugated to anti-human IgG1 or anti-human IgG4. Upon laser irradiation of the beads-target complexes at 680 nm, short-lived singlet oxygen molecules produced by the Donor beads can reach the Acceptor beads in proximity to generate an amplified chemiluminescent signal at 615 nm. The intensity of light emission is proportional to the level of the specific IgG subclass detected.

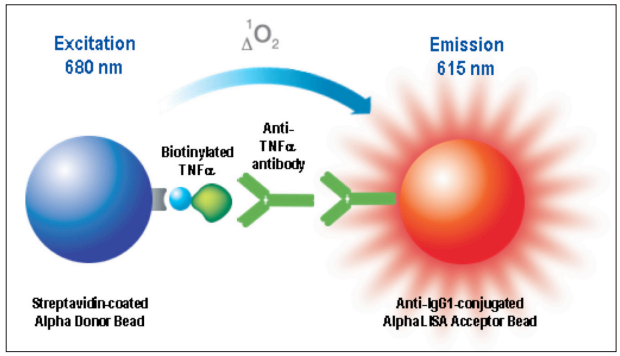

Figure 1. Schematic representation of the AlphaLISA detection of anti-TNFα antibody, an immunoglobulin G subclass 1 antibody.

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Development of Anti-human IgG Assay

Reagents needed for this assay:

Anti-IgG1 AlphaLISA Acceptor beads	PerkinElmer # AL141
Anti-IgG4 AlphaLISA Acceptor beads	PerkinElmer # AL142
Alpha Streptavidin Donor beads	PerkinElmer # 6760002
AlphaLISA HiBlock buffer, 10X, 10 mL	PerkinElmer # AL004C
White opaque OptiPlate™-384	PerkinElmer # 6007290
TopSeal™-A film	PerkinElmer # 6050195
Anti-hTNFα-IgG1	InvivoGen # htnfa-mab1
Anti-hTNFα-IgG4	InvivoGen # htnfa-mab4
Recombinant Human TNFα*	PeproTech # 300-01A
Primate Cynomolgus Monkey Serum (pool of sera)	Primus Bio-Ressources
Rat Sprague Dawley Serum (pool of sera)	Bioreclamation # RATSRM
Mouse BALB/C Serum (pool of sera)	Bioreclamation # MSESRM-BALB
ChromaLink Biotin (DMF Soluble)	Solulink # B-1001

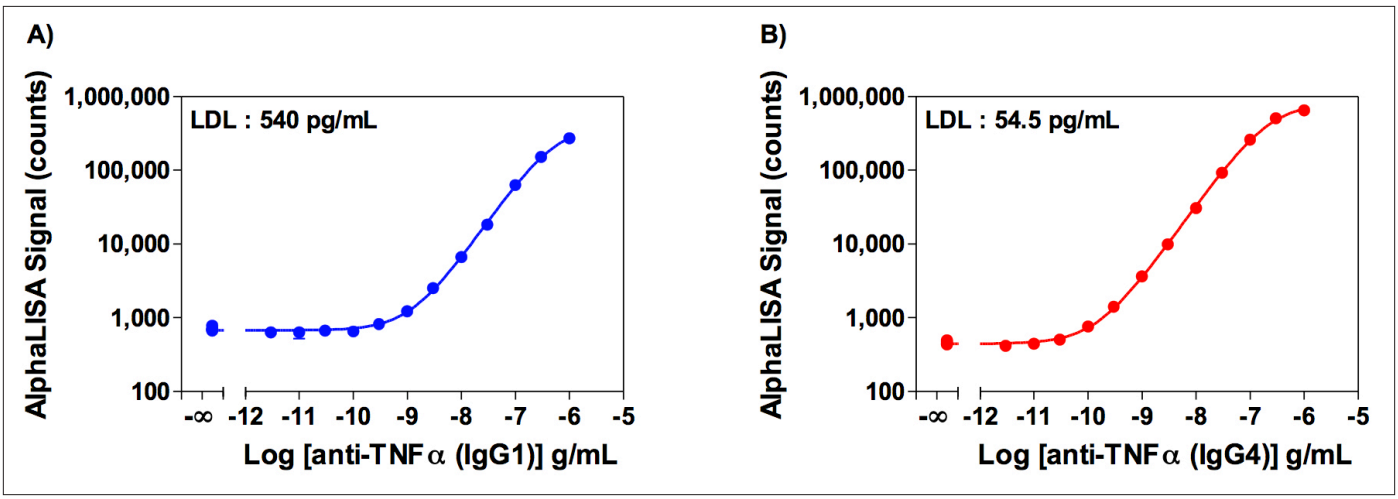
*Human TNFα was biotinylated with Chromalink Biotin, using a standard biotinylation procedure.

Standard Protocol

- Prepare dilutions of sera at 10%, 50% and 25% for monkey, rat and mouse, respectively, by appropriate dilution in 1X HiBlock buffer.

  Note: In previous experiments, different dilutions of sera in assay buffer were tested to determine which dilutions were required to provide acceptable assay accuracy (70 – 130% recovery).
- Three sets of samples are prepared:
  - **Standards:** Dilute human anti-TNFα IgG1 and IgG4 in pre-diluted sera to generate standard curves.
  - **Spiked samples:** Prepare 100X spiked samples at 10, 1 and 0.1 µg/mL in 100% serum. Dilute each set of spiked samples 1/100 with pre-diluted sera to obtain 100, 10 and 1 ng/mL final concentrations.
  - **Samples for dilutional linearity:** Starting from the spiked sample at 100 ng/mL, prepare a 2-fold serial dilution, from undiluted to 16-fold dilution, with pre-diluted sera.
- Prepare 5X anti-IgG1 and anti-IgG4 AlphaLISA Acceptor beads and biotinylated TNFα at 50 µg/mL and 5 nM, respectively, in 1X HiBlock buffer. Final concentrations are, respectively, 10 µg/mL and 1 nM in 50 µL total assay volume.
- Add to the wells of a white OptiPlate-384:
  - 5 µL of human anti-TNFα IgG1 or IgG4 dilutions for standard curves OR 5 µL of spiked samples OR 5 µL of dilutions for linearity, in triplicate
  - 10 µL of 5X Acceptor beads
- Incubate 30 min at RT.
- Add 10 µL of 5X biotinylated TNFα.
- Incubate 60 min at RT.
- Prepare 2X Streptavidin Donor beads at 80 µg/mL in 1X HiBlock buffer, in subdued light (final concentration of 40 µg/mL in 50 µL total assay volume).
- Add 25 µL of Donor beads in subdued light.
- Cover with TopSeal-A film and incubate in the dark for 30 min at RT.
- Read signal in Alpha mode with the EnVision® or EnSpire® readers.

Experiment 1: Titration Curves of anti-TNFα Antibodies



Anti-TNFα titration curves were performed by incubating 5 µL of anti-TNFα antibodies from IgG1 (A) or IgG4 (B) subclasses (diluted with 10% Monkey serum from 1E-6 to 3E-12 g/mL) with 10 µL of a 5X solution (50 µg/mL) of anti-IgG1 or anti-IgG4 Acceptor Beads for 30 min at RT. Then, an addition of 10 µL of a 5X solution (5 nM) of biotinylated TNFα was followed by a 60 min incubation at RT. Finally, 25 µL of a 2X solution (80 µg/mL) of Streptavidin Donor beads were added and signal was read after 30 min. Lower Detection Limit (LDL) was calculated by interpolating the average background counts (12 wells without anti-TNFα antibodies) + 3 x standard deviation value (average background counts + (3xSD)) on the standard curve.

Experiment 2: Dilutional Linearity

Table A

Dilution Factor	% Recovery		
	Monkey Serum	Mouse Serum	Rat Serum
1	100	100	100
2	99	94	95
4	100	89	92
8	99	86	91
16	104	88	93

Table B

Dilution Factor	% Recovery		
	Monkey Serum	Mouse Serum	Rat Serum
1	100	100	100
2	103	98	94
4	105	96	88
8	104	94	86
16	103	93	83

Dilutional linearity was determined by serial dilutions of a pre-diluted pool of sera spiked with 100 ng/mL of human anti-TNFα IgG1 or IgG4. The recovery was calculated using the 100 ng/mL sample as the 100% value. The average recovery from two independent measurements is reported. Table A and table B show results for IgG1 and IgG4, respectively.

Experiment 3: Spike Recovery

Table A

Spike (ng/mL)	% Recovery		
	Monkey Serum	Mouse Serum	Rat Serum
100	80	90	92
10	77	85	88
1	71	90	92

Table B

Spike (ng/mL)	% Recovery		
	Monkey Serum	Mouse Serum	Rat Serum
100	82	95	99
10	76	87	87
1	77	89	92

Three known concentrations of human anti-TNFα IgG1 or IgG4 were spiked in a pre-diluted pool of sera. The % Recovery was calculated by comparison to the theoretical concentration of antibody. The average recovery from two independent measurements is reported. Table A and table B show results for IgG1 and IgG4, respectively.

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