Human IgG Subclass Isotype ELISA

SOP 23116 Rev. 02

Biopharmaceutical Development Program

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1.0 Purpose

This procedure describes a qualitative method used to determine the subclass of Human IgG products manufactured at the Biopharmaceutical Development Program (BDP), as well as to distinguish the light chain as kappa or lambda by ELISA.

2.0 Scope

This procedure applies to Process Analytics personnel who will perform Human IgG Subclass testing.

3.0 Authority and Responsibility

- 3.1 The Director, Process Analytics (PA) has the authority to define this procedure.
- 3.2 PA is responsible for training on this procedure and documenting this training to Biopharmaceutical Quality Assurance (BQA).
- 3.3 PA personnel are responsible for the performance of this procedure.
- 3.4 BQA is responsible for quality oversight of this operation.

4.0 Equipment, Materials and Controlled Reagents

- 4.1 Equipment, Materials
 - 4.1.1 Labsystems iEMS Microtiter Plate Reader MF with Ascent software version 2.4.2, Model Number 1401, BDP MEF 66160, or BDP approved equivalent. Operate in accordance with **SOP 22100**, *Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser.*
 - 4.1.2 Calibrated multi-channel pipettor.
 - 4.1.3 Reagent reservoirs, BDP PN **BDP**, or BDP approved equivalent.
 - 4.1.4 Calibrated pipettors 2-200 µL and 100-1000 µL.

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- 4.1.5 Pipet Tips, 250 μL, BDP PN and 1-1000 μL, BDP PN approved equivalent.
- 4.1.6 Microcentrifuge tubes 1.5 mL, BDP PN _____, or BDP approved equivalent.
- 4.1.7 Low-lint or lint-free absorbent paper, BDP PN _____, or BDP approved equivalent.
- 4.1.8 1 Liter Graduated Cylinder for wash solution preparation.
- 4.1.9 Direct-Q or Milli-Q H₂O.
- 4.2 Controlled Reagents
 - 4.2.1 Human IgG Subclass ELISA Kit, BDP PN
 - 4.2.2 IgG1, Lambda, Human, BDP PN , or BDP approved equivalent.
 - 4.2.3 IgG1, Kappa, Human, BDP PN
 - 4.2.4 Anti-Human Lambda Light Chain (FITC), BDP PN , or BDP approved equivalent.
 - 4.2.5 Anti-Human Kappa Light Chain (FITC), BDP PN

5.0 Procedure

- 5.1 Allow Human IgG Subclass ELISA kit and standards to warm up to room temperature for 15 45 minutes.
- 5.2 Wash buffer dilute 25X stock to 1X with Direct-Q or Milli-Q H₂O. Record preparation in the BQC solution logbook in accordance with **SOP 22702**, *Solutions Used in BQC*.
- 5.3 Conjugate Solution Dilute 50X concentrated peroxidase-anti-human IgG in diluent buffer at a ratio of 1:50. Example: Add 0.1 mL of conjugate to 5 mL of diluent for each 96 well plate. Do not prepare more than is needed for each experiment (100 μL per well will be needed).
- 5.4 Human IgG Subclass Standards Reconstitute lyophilized standard with 2 mL of diluent buffer. Mix gently until visually clear.
- 5.5 Light Chain Standards Dilute Human Kappa Light Chain (BDP PN) and Human Lambda Light Chain (BDP PN) separately to achieve a final concentration of 10 μg/mL using diluent buffer. Prepare at least 200 μL of each standard for each experiment. Record dilution on Form 23116-01. Refer to the manufacturer's certificates of analysis (CofA) for the protein concentration.
- 5.6 Light Chain Antibodies Dilute Anti-human Lambda Light Chain (FITC) and Anti-human Kappa Light Chain (FITC) separately at 1:1000 using Diluent buffer. Dilute 1:10 then dilute 1:100 to achieve a total dilution of 1:1000. Record dilution on Form 23116-01.
- 5.7 Sample Preparation
 - 5.7.1 Dilute test article to 50 μg/mL in Diluent buffer. Example: Add 41.7 μL of 1.2 mg/mL test article to 958.3 μL sample diluent to achieve 50 μg/mL. Record dilution on Form 23116-01.
 - 5.7.2 Label 6 tubes #1, #2, #3, #4, K, L

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5.7.3 Add Diluent buffer and diluted test article from step 5.7.1 to the tubes as outlined in the following table.

Tube	Target Concentration (µg/mL)	Subclass to Test Against	Volume of 50 μg/mL diluted sample (μL)	Volume of Sample Diluent (µL)
#1	6.86	lgG ₁	137.2	862.8
#2	2.66	lgG ₂	53.2	946.8
#3	0.67	lgG ₃	13.4	986.6
#4	0.38	lgG ₄	7.6	992.4
K	10	LC Kappa	200	800
L	10	LC Lambda	200	800

5.7.4 Place the needed number of microtiter strips in plate frame. Samples, Blanks and Standards must be tested in duplicate. See example template below. Record actual plate layout on ELISA Plate Template – Form 23116-01.

Figure 1 Example ELISA Plate Layout

	1	2	3	4	5	6	1	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank						
В	Blank	Blank	Blank	Blank	Blank	Blank						
С	Sample 10 ug/mL	Sample 10 ug/mL	Sample 6.86 ug/mL	Sample 2.66 ug/mL	Sample 0.67 ug/mL	Sample 0 38 ug/mL						
D	Sample 10 ug/mL	Sample 10 ug/mL	Sample 6.86 ug/mL	Sample 2.66 ug/mL	Sample 0.67 ug/mL	Sample 0 38 ug/mL						
E	Kappa Light Chain STD 10 ug/mL	Lambda Light Chain STD 10 ug/mL	Subclass STD 2E 6.86 ug/mL IgG1	Subclass STD 2E 2.66 ug/mL IgG2	Subclass STD 2E 0 67 ug/mL IgG3	Subclass STD 2E 0 38 ug/mL IgG4						
F	Kappa Light Chain STD 10 ug/mL	Lambda Light Chain STD 10 ug/mL	Subclass STD 2E 6.86 ug/mL IgG1	Subclass STD 2E 2.66 ug/mL IgG2	Subclass STD 2E 0 67 ug/mL IgG3	Subclass STD 2E 0 38 ug/mL IgG4						
G												
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Anti-Kappa Anti-Lambda Light Chain Ab Light Chain Ab	Anti-IgG1 Ab	Anti-IgG2 Ab	Anti-IgG3 Ab	Anti-IgG4 Ab
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- 5.7.5 Add 50 μL of primary antibody to the appropriate wells according to the ELISA plate layout (see Figure 1: Example ELISA Plate Layout). Subclass antibodies are prediluted: IgG1, IgG2, IgG3, IgG4; for the Anti-Kappa and Anti-Lambda antibodies, use the dilutions (1:1000) made from step 5.6.
- 5.7.6 Sample/Control/Standards Dispense 50 μL of diluted samples, Human IgG standards, and Light chain standards to their respective wells according to 96-well template. For Blank wells, use 50 μL of diluent buffer.
- 5.7.7 Incubate at room temperature 20-40 minutes.
- 5.7.8 Plate washing Wash the plate strips by filling each well with Wash Buffer (400 μL) using a multi-channel pipette. Decant plate contents and repeat the process three times for a total of four washes. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
- 5.7.9 Conjugate dispensing Dispense 100 µL of diluted conjugate solution to each well.
- 5.7.10 Incubate for 20-40 minutes at room temperature.
- 5.7.11 Plate washing Repeat as performed in step 5.7.8
- 5.7.12 Substrate dispensing Dispense 100 μ L of the TMB substrate solution to each well.
- 5.7.13 Incubate for 8-12 minutes at room temperature protected from light.
- 5.7.14 Stopping Reagent Dispense 100 μL of stopping reagent into each well. Be sure to work quickly to obtain a uniform stop time for each well. Mix each well by pipeting up and down 3-5 times.
- 5.7.15 Plate reading Determine the optical density (OD) of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
- 5.8 Calculation of Results
 - 5.8.1 <u>Averages and Relative Standard Deviations (RSDs)</u> using Microsoft Excel, input the corrected (450nm – 540 or 570 nm) OD values and calculate the average, standard deviation (both Excel formula functions), and RSD% ((standard deviation / average OD) x 100) of the duplicate OD values for the standards, samples and blanks.
 - 5.8.2 <u>Correlation Percentage</u> Divide the mean absorbance of the test article for each IgG subclass and each light chain type by the absorbance of the corresponding standard and multiply by 100% to obtain the Correlation Percentage. Example: 2.195 (sample) / 2.286 (standard) x 100% = 96.0%. Enter results into the table on Form 23116-01.

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- 5.8.3 <u>Heavy Chain isotype determination</u> The correlation percentage of the test article that is within 70-200% of the corresponding IgG subclass standard is to be reported as that isotype. Example: Isotype IgG1 = 90% would be reported as "Isotype = IgG1."
- 5.8.4 <u>Light Chain isotype determination</u> The correlation percentage of the test article that is within 70-200% of the corresponding Light Chain standard is to be reported as that Light Chain. Example: Light Chain Kappa = 90% would be reported as "Light Chain = Kappa."

6.0 Data Analysis and Acceptance Criteria

- 6.1 <u>Test Article Correlation Percentage</u> Only one IgG isotype and one light chain can be within 70-200% of a standard (IgG₁, IgG₂, IgG₃, IgG₄, kappa light chain, lambda light chain). If more than one IgG isotype or more than one light chain is within 70-200%, then that portion of the test is to be reported as undetermined. Example: Isotype IgG1 = 90% and Isotype IgG2 = 70% would be reported as "Isotype = undetermined."
- 6.2 The RSD% must not exceed 25% for standards or test articles with an absorbance at 450 nm above 0.2 au.
- 6.3 If the above criteria are not met, the assay is invalid and must be repeated.

7.0 Documentation

- 7.1 Print and attach the ELISA worksheet form (Form 23116-01), raw data, and calculations to the QC Test Request Form.
- 8.0 References and Related Documents
 - **SOP 22100** Operation of the Labsystems iEMS Microtiter Plate Reader/Dispenser

SOP 22702 Solutions Used in BQC

Form 23116-01 Human IgG Subclass ELISA Worksheet

9.0 Attachments

9.1 Attachment 1 Human IgG Subclass ELISA Product Insert

10.0 Change Summary



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Attachment 1 Human IgG Subclass ELISA Product Insert

							Human IgG Subclass Profile	
							192 Tests	
		•					Technical Data Sheet	
Ca	atalog # 99100	00						
*N	ata: A lattar at the	and of the le	t mmhar ai an	ifias on addis	tional poaltoo	ing of this come	1.4+	
14	ote. A letter at the c	chie of the lo	t number sign	incs an addi	uonai packag	ing of this same	; IOL	
Int The qua if t	ended Use and Ma e Human IgG Sub antitative measurem he recommended as	aterials Profi toclass Profi tont of Hum ssay procedu	vided le ELISA Ki an IgG1, IgG2 ire, storage an	t contains c 2, IgG3, and d handling o	components a IgG4 subclas of materials ar	equired to con sses. Sufficient o e followed as sp	struct an enzyme-linked immunoassay for the specific an quantities of reagents are provided to yield 2 plates of 96 well beeified on this insert.	
1.	Antibody:	mAb Anti-Human leG1						
		mAb Ant	i-Human IgO	22				
		mAb Ant	i-Human IgC	33				
	Form	I imid 4	i-Human Ige	each vial				
	Storage:	Store at 2	to 8°C until e	xpiration dat	e.			
2.	Control:	Human S	erum Contro	,				
	Form:	Lyophiliz	ed, 2 vials. Co	ontains 0.1%	sodium azid	e.		
	Storage: Reconstitution:	Store at 2 Reconstitu	to S'C until e	xpiration dat	c. with 1.0 ml	of Diluent Buff	er. Swirl or mix cently and allow to sit for 10 minutes to assure	
	reconstitution.	complete	reconstitution	. Use control	within 1 hor	ir of reconstituti	on.	
	Ranges:	IgG1 (1.7	$7 - 2.3 \mu\text{g/mL}$)				
		IgG2 (0.5	5 – 1.3 µg/mL)				
		IgG3 (0.1)	$13 - 0.3 \ \mu g/m$	L)				
3.	Standard:	Human I	G Subclass	Standard				
	Form:	Lyophiliz	ed, 2 vials. Co	ntains 0.1%	sodium azide	2.		
	Storage:	Store at 2	2 to 8°C.					
	Decementation		ute each lyophilized standard vial with 1.0 mL of Diluent Buffer. Swirl or mix gently and allow to sit for 10 minutes to					
	Reconstitution.	ensure con	ute each lyoph mplete recons	nilized standa titution. Use	ard vial with i standard with	1.0 mL of Dilue hin 1 hour of rec	nt Buffer. Swirl or mix gently and allow to sit for 10 minutes constitution.	
	Standard Curve:	Reconstitu ensure con To genera 1.0 mL, 1 0.76 µg/m Standard (te each lyoph mplete recons te a 6-point st the concentra IL of IgG4. B (ug/mL)	titution. Use andard curve tions of the elow is the c	ard vial with 1 standard with e, make serial standard are concentration	1.0 mL of Dilue nin 1 hour of rea dilutions of the a 13.72 μg/mL of each IgG wh	nt Buffer. Swirl or mix gently and allow to sit for 10 minutes constitution. e standard using the Diluent Buffer. When reconstituted in of IgG1, 5.32 µg/mL of IgG2, 1.34 µg/mL of IgG3, an en diluted serially in half.	
	Standard Curve:	Reconstitu ensure con To genera 1.0 mL, 0.76 µg/m Standard (the each lyoph mplete recons the a 6-point st the concentra aL of IgG4. B (µg/mL)	nilized standar titution. Use andard curve tions of the elow is the c	ard vial with i standard with e, make serial standard are concentration	1.0 mL of Dilue nin 1 hour of rec dilutions of the = 13.72 µg/mL of each IgG wh	nt Buffer. Swirl or mix gently and allow to sit for 10 minutes to constitution. z standard using the Diluent Buffer. When reconstituted in of IgG1, 5.32 µg/mL of IgG2, 1.34 µg/mL of IgG3, an en diluted serially in half.	
	Standard Curve:	Reconstitu ensure con To genera 1.0 mL, 1 0.76 µg/m Standard (te each lyoph mplete recons te a 6-point st the concentra iL of IgG4. B (µg/mL) IgG1 13.72	nilized standa titution. Use andard curve tions of the telow is the c IgG2 5.32	ard vial with i standard with c, make serial standard are concentration IgG3 1.34	1.0 mL of Dilue nin 1 hour of rec dilutions of the e 13.72 µg/mL of each IgG wh IgG4 0.76	nt Buffer. Swirl or mix gently and allow to sit for 10 minutes constitution. constitution. catandard using the Diluent Buffer. When reconstituted in of IgG1, 5.32 µg/mL of IgG2, 1.34 µg/mL of IgG3, an en diluted serially in half.	
	Standard Curve:	Reconstitu ensure con To genera 1.0 mL, 1 0.76 µg/m Standard o Neat 1.2	the each lyoph mplete reconsist the concentration of IgG4. B (µg/mL) IgG1 13.72 6.86	hilized stands titution. Use andard curve tions of the elow is the c IgG2 5.32 2.66	ard vial with i standard with e, make serial standard are concentration IgG3 1.34 0.67	1.0 mL of Dilue in 1 hour of rec dilutions of the e 13.72 µg/mL of each IgG wh IgG4 0.76 0.38	nt Buffer. Swirl or mix gently and allow to sit for 10 minutes to constitution. Estandard using the Diluent Buffer. When reconstituted in of IgG1, 5.32 µg/mL of IgG2, 1.34 µg/mL of IgG3, and en diluted serially in half.	
	Standard Curve:	Reconstitu ensure con To genera 1.0 mL, 1 0.76 µg/m Standard (Neat 1:2 1:4	te each lyoph mplete recons te a 6-point st the concentra L of IgG4. B (µg/mL) IgG1 13.72 6.86 3.43	ilized stands titution. Use andard curve tions of the elow is the c IgG2 5.32 2.66 1 33	ard vial with 1 standard with e, make serial standard are concentration IgG3 1.34 0.67 0.34	1.0 mL of Dilue in 1 hour of rec dilutions of the e 13.72 µg/mL of each IgG wh IgG4 0.76 0.38 0.19	nt Buffer. Swirl or mix gently and allow to sit for 10 minutes to constitution. standard using the Diluent Buffer. When reconstituted in of IgG1, 5.32 µg/mL of IgG2, 1.34 µg/mL of IgG3, and en diluted serially in half.	
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	Standard Curve:	Reconstituent ensure con To generat 1.0 mL, 1 0.76 µg/m Standard of Neat 1:2 1:4 1:8 1:16 1:32	te each lyoph mplete recons te a 6-point st the concentra dL of IgG4. B (µg/mL) 13.72 6.86 3.43 1.72 0.86 0.43	illized stands titution. Use andard curve tions of the elow is the c IgG2 5.32 2.66 1.33 0.67 0.33 0.17	ard vial with 1 standard with c, make serial standard are concentration IgG3 1.34 0.67 0.34 0.17 0.084 0.042	1.0 mL of Dilue in 1 hour of rec dilutions of the 13.72 µg/mL of each IgG wh IgG4 0.76 0.38 0.19 0.095 0.048 0.024	nt Buffer, Swirl or mix gently and allow to sit for 10 minutes to constitution. standard using the Diluent Buffer. When reconstituted in of IgG1, 5.32 µg/mL of IgG2, 1.34 µg/mL of IgG3, and en diluted serially in half.	
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Human IgG Subclass Isotype ELISA

SOP 23116 Rev. 02

Biopharmaceutical Development Program

Attachment 1 (Continued)

Additional Materials Required

Pipettes and timer.

- · Microplate reader with a detector that can measure absorbance at 450 nm.
- 1 L graduated cylinder; plate washer or wash bottle.
- Polypropylene tubes for standards and sample dilutions, if needed.

Principle of the Assay

This kit is a sandwich type ELISA using a horseradish peroxidase detection system. A coated microtiter plate captures monoclonal reagents which are specific to the various human IgG subclasses. The monoclonal antibodies in turn capture the human IgG subclasses, for which they are specific, out of the serum sample. These monoclonal antibodies have been characterized in a IUIS/WHO study. The captured human IgG is then labeled by a horseradish-peroxidase anti-human IgG reagent. The detection signal is then generated in proportion to the amount of human subclass antibody.

Recommended Assay Procedure

 Prior to use, allow the kit to warm to room temperature. Remove the number of strip-wells according to your design plan. It is suggested to run all samples in duplicate.

Example of experimental plate plan setup for IgG1 only:

Standard IgG1

0	0	Control	Control			
Neat	Neat	Sample	Sample			
1:2	1:2	Sample	Sample			
1:4	1:4	Sample	Sample			
1:8	1:8	Sample	Sample			
1:16	1:16	Sample	Sample			
1:32	1:32	Sample	Sample			
		Sample	Sample			

- Add 50 µL of the appropriate human subclass specific antibody (for example, MAb Anti-Human IgG1) to each well except for zero wells. For the zero wells, add 50 µL of diluted serum samples and then, add 50 µL of the Diluent Buffer.
- 3. Then, add 50 µL of diluted serum samples, standards, and the ready-to-use *Human Serum Control* to their respective wells. (Suggested dilution for human sample is 1:2500 as a starting point. However, it is up to the investigator to determine the optimal dilution.) Gently tap the plate on the side 10 times to mix. Incubate at room temperature for 30 min.
- 4. Remove contents by inverting the plate. Wash four times by adding 400 µL of diluted Wash Buffer into each well. Let soak for 15 to 30 seconds, then remove by inverting the plate and tapping on absorbent paper to remove excess liquid.
- 5. Add 100 µL of diluted Peroxidase Anti-Human IgG solution into each well. Incubate at room temperature for 30 min.
- 6. Remove contents by inverting the plate. Wash four times using the method in Step 4.
- Add 100 μL of the ready-to-use *TMB Solution* into each well. The liquid in the wells will begin to turn blue. Incubate at room temperature and in the dark for 10 min.
- Quickly add 100 μL of *Stop Solution* into each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
- Measure absorbance at 450 nm (reference absorbance: 650 nm) within 1 hour of adding the Stop Solution. Calculate results using a log-log or 4-parameter curve fit.

	Ex	planation	of symbols
Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch oode
RUO	Research Use Only	IVD	In vitro diagnostic medical device
X	Use by	ł	Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
Ø	Protect from light	Â	Consult accompanying documents
Ti	Directs the user to consult	instructions fo	or use (IFU), accompanying the product.

For Research Use Only. Caution: Not for human or animal therapeutic or diagnostic use.

PI991000 (Rev 10/10)

DCC-10-2297

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