Table of Contents

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 [redacted], or BDP approved equivalent.

4.1.4 Calibrated pipettors 2-200 µL and 100-1000 µL.

4.1.5 Pipet Tips, 250 µL, BDP PN [redacted] and 1-1000 µL, BDP PN [redacted], or BDP approved equivalent.

4.1.6 Microcentrifuge tubes 1.5 mL, BDP PN [redacted], or BDP approved equivalent.

4.1.7 Low-lint or lint-free absorbent paper, BDP PN [redacted], or BDP approved equivalent.

4.1.8 1 Liter Graduated Cylinder for wash solution preparation.

4.1.9 Direct-Q or Milli-Q H_2O.

4.2 Controlled Reagents

4.2.1 Human IgG Subclass ELISA Kit, BDP PN [redacted], or BDP approved equivalent.

4.2.2 IgG1, Lambda, Human, BDP PN [redacted], or BDP approved equivalent.

4.2.3 IgG1, Kappa, Human, BDP PN [redacted], or BDP approved equivalent.

4.2.4 Anti-Human Lambda Light Chain (FITC), BDP PN [redacted], or BDP approved equivalent.

4.2.5 Anti-Human Kappa Light Chain (FITC), BDP PN [redacted], or BDP approved equivalent.

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 H_2O. 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 [redacted]) and Human Lambda Light Chain (BDP PN [redacted]) 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

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	IgG_1	137.2	862.8
#2	2.66	IgG_2	53.2	946.8
#3	0.67	IgG_3	13.4	986.6
#4	0.38	IgG_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	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank						
B	Blank	Blank	Blank	Blank	Blank	Blank						
C	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												
H												

Anti-Kappa Light Chain Ab	Anti-Lambda Light Chain Ab	Anti-IgG1 Ab	Anti-IgG2 Ab	Anti-IgG3 Ab	Anti-IgG4 Ab

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 pre-diluted: 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 µL 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 Averages and Relative Standard Deviations (RSDs) – 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 Correlation Percentage - 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.

5.8.3 Heavy Chain isotype determination – 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 Light Chain isotype determination – 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 Test Article Correlation Percentage - Only one IgG isotype and one light chain can be within 70-200% of a standard (IgG_1, IgG_2, IgG_3, IgG_4, 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

[redacted]		

Attachment 1

Human IgG Subclass ELISA Product Insert

[redacted]

Human IgG Subclass Profile

192 Tests

Technical Data Sheet

Catalog #: 991000 [redacted]

*Note: A letter at the end of the lot number signifies an additional packaging of this same lot.

Intended Use and Materials Provided

The Human IgG Subclass Profile ELISA Kit contains components required to construct an enzyme-linked immunoassay for the specific and quantitative measurement of Human IgG1, IgG2, IgG3, and IgG4 subclasses. Sufficient quantities of reagents are provided to yield 2 plates of 96 wells if the recommended assay procedure, storage and handling of materials are followed as specified on this insert.

1. **Antibody:** **mAb Anti-Human IgG1** [redacted]
   mAb Anti-Human IgG2
   mAb Anti-Human IgG3
   mAb Anti-Human IgG4
   Form: Liquid, 4 vial x 2.5 mL each vial
   Storage: Store at 2 to 8°C until expiration date.
2. **Control:** **Human Serum Contro** [redacted]
   Form: Lyophilized, 2 vials. Contains 0.1% sodium azide.
   Storage: Store at 2 to 8°C until expiration date.
   Reconstitution: Reconstitute the lyophilized control with 1.0 mL of Diluent Buffer. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use control within 1 hour of reconstitution.
   Ranges: IgG1 (1.7 – 2.3 µg/mL)
   IgG2 (0.5 – 1.3 µg/mL)
   IgG3 (0.13 – 0.3 µg/mL)
   IgG4 (0.1 – 0.2 µg/mL)
3. **Standard:** **Human IgG Subclass Standard** [redacted]
   Form: Lyophilized, 2 vials. Contains 0.1% sodium azide.
   Storage: Store at 2 to 8°C.
   Reconstitution: Reconstitute each lyophilized standard vial with 1.0 mL of Diluent Buffer. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.
   Standard Curve: To generate a 6-point standard curve, make serial dilutions of the standard using the Diluent Buffer. When reconstituted in 1.0 mL, the concentrations of the standard are 13.72 µg/mL of IgG1, 5.32 µg/mL of IgG2, 1.34 µg/mL of IgG3, and 0.76 µg/mL of IgG4. Below is the concentration of each IgG when diluted serially in half.

Standard (µg/mL)

	IgG1	IgG2	IgG3	IgG4
Neat	13.72	5.32	1.34	0.76
1:2	6.86	2.66	0.67	0.38
1:4	3.43	1.33	0.34	0.19
1:8	1.72	0.67	0.17	0.095
1:16	0.86	0.33	0.084	0.048
1:32	0.43	0.17	0.042	0.024

4. **Secondary antibody:** **Peroxidase Anti-Human IgG (Part # 50177HK Lot #: 844411)**
   Form: Liquid, 1 vial x 0.5 mL (50X Concentrate)
   Storage: Store at 2 to 8°C until expiration date.
   Recommended Dilution: Dilute concentrated Peroxidase-Anti-Human IgG in Diluent Buffer at a ratio of 1:50. For example, add 0.22 mL of conjugate to 10.78 mL of diluent for each 96 well plate. Do not prepare more diluted Anti-Human IgG solution than is needed. Discard any unused portion.
5. **Chromogen:** **TMB Solution** [redacted]
   Form: 1 vial x 25 mL
   Stop Solution: **Stop Solution** [redacted]
   Form: 1 vial x 25 mL
6. **Diluent:** **Diluent Buffer** [redacted]
   Form: 1 vial x 135 mL
7. **Wash Buffer:** **Wash Buffer Concentrate (25X)** [redacted]
   Form: 100 mL bottle
   Reconstitution: Dilute 1 volume of the 25x wash buffer concentrate with 24 volumes of deionized water (ie. 100 mL may be diluted up to 2.5 liters).
8. **Plate:** **IgG Antibody-Coated Wells, 12 x 8 Well Strips - 2 Plates** [redacted]

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

1. 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								

2. 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.
7. 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**.
8. 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.
9. 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.

Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

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

[redacted]