KWALITY PHARMACEUTICALS LIMITED

1-A, INDUSTRIAL AREA, RAJA KA BAGH, TEHSIL NURPUR, KANGRA- 176201 (INDIA)

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QUALITY CONTROL DEPARTMENT

FINISH PRODUCT SPECIFICATION

PRODUCT NAME	**CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/ VIAL**		
GENRIC NAME	CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/ VIAL		
PRODUCT CODE	CY/006	**EFFECTIVE DATE**	21/06/23
GRADE	BP-2022	**REVIEW DATE**	20/06/25
SPECIFICATION NO.	KPL/SPC/IN/105-00	**VERSION NO.**	00
STP NO.	KPL/STP/IN/105-00	**SUPERSEDES NO.**	Nil
Page No.	1 of 2		

Primary pack details	Clear colourless transparent USP type I glass vial.
Label Claim	Cyclophosphamide BP 200 mg/vial.
Storage Condition	Stored below 30°C, protect from moisture and light.
Proposed Shelf Life	36 Month
Sample Quantity	40 Vials

Sr. NO.	TESTS	SPECIFICATION	REFERENCE
1.	**Description:**		
	Before reconstitution	White or off white lyophilized cake filled in clear colorless glass vial USP type I.	In House
	After reconstitution	Clear colourless solution is produced after reconstitution with 10ml water for injection.	In House
2.	**Identification**		
	A. By IR	The infrared absorption spectrum of the filtrate is concordant with the reference spectrum of Cyclophosphamide.	BP Monograph
	B. By Chemically	A white precipitate is produced which is insoluble in nitric acid but soluble in 5 M ammonia from which it is re-precipitated on the addition of nitric acid	BP Monograph
3.	**Acidity (pH)**	Between 3.0 to 6.0	BP Monograph
4.	**Average Weight**	415mg ±10% (373.5mg -456.5mg)	BP Monograph (Appendix XIIC1)
5.	**Uniformity of Content: By Method A**	85.0% to 115.0% of average content.	BP Monograph (Appendix XIIC3)
6.	**Sterility (By Membrane Filtration)**	Sterile	BP Monograph (Appendix XVI A)
7.	**Particulate Contamination:**		BP Monograph (Appendix XIII A)
	Visible Particle	It should be free from any type of visual	

PREPARED BY (SIGN/ DATE) (QC PERSONNEL)	CHECKED BY (SIGN/ DATE) (HEAD – QC)	APPROVED BY (SIGN/ DATE) (HEAD QA)	
21/06/23	21/06/23	21/06/23	

ANNEXURE NO.: QC071/A01-01

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Sr. NO.	TESTS	SPECIFICATION	REFERENCE
		particles	
	Sub visible particles, By Light Obscuration Particle Count Test.	For 10 µm to less than 25 µm - NMT 6000 particles For 25 µm or greater - 600 particles	
8.	**Bacterial Endotoxins Test**	NMT 0.0625 EU/mg of Cyclophosphamide.	BP Monograph (Appendix XIVC)
9.	**Related substances: (By TLC)**	Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) (1%)	BP Monograph
10.	**Assay: Each vial contain: (By Titration)**		
	Active ingredient	**Label Claim** / **Limit**	BP Monograph
	Cyclophosphamide (Anhydrous) BP	200 mg / 92.5% to 107.5% (185.0 mg to 215 mg)	

REVISION HISTORY:

Sr. No.	Change control No.	Revision No.	Reason for Revision
01	CC/23/06/QC/039	00	New Specification

END OF DOCUMENT

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QUALITY CONTROL DEPARTMENT

BULK SPECIFICATION

PRODUCT NAME	**CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/ VIAL**		
GENRIC NAME	CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/ VIAL		
PRODUCT CODE	CY/006	**EFFECTIVE DATE**	21/06/23
GRADE	BP-2022	**REVIEW DATE**	20/06/25
SPECIFICATION NO.	KPL/SPC/BI/105-00	**VERSION NO.**	00
STP NO.	KPL/STP/IN/105-00	**SUPERSEDES NO.**	Nil
Page No.	1 of 1		

Shelf Life	36 Month
Storage Condition	Stored below 30°C, protect from moisture and light.
Sample Quantity	20ml

Sr. NO.	TESTS	SPECIFICATION		
1.	**Description**	Clear colorless solution.		
2.	**Identification: By HPLC**	The retention time of the major peak of the sample solution correspond to that of the standard solution, as obtained in the assay		
3.	**Acidity (pH)**	Between 3.0 to 6.0		
4.	**Assay: Each vial contains**			
	Active Ingredient	**Label Claim**	**Release Limit**	**Shelf Limit**
	Cyclophosphamide (Anhydrous) BP	200 mg	194.2 mg to 215.0mg 97.1% to 107.5%	185.0 mg to 215.0mg 92.5% to 107.5%

REVISION IIISTORY:

Sr. No.	Change control No.	Revision No.	Reason for Revision
01	CC/23/06/QC/039	00	New Specification

END OF DOCUMENT

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STABILITY PRODUCT SPECIFICATION

PRODUCT NAME	**CYCLOPHOSPHAMIDE FOR INJECTION BP 200MG/VIAL**		
GENRIC NAME	CYCLOPHOSPHAMIDE FOR INJECTION BP 200MG/ VIAL		
PRODUCT CODE	CY/006	**EFFECTIVE DATE**	21/06/23
GRADE	BP-2022	**REVIEW DATE**	20/06/25
SPECIFICATION NO.	KPL/SPC/SI/104-00	**VERSION NO.**	00
STP NO.	KPL/STP/IN/104-00	**SUPERSEDES NO.**	Nil
Page No.	1 of 2		

Primary pack details	Clear colourless transparent USP type I glass vial
Label Claim	Cyclophosphamide BP 200mg/vial
Storage Condition	Stored below 30°C, protect from moisture and light.
Proposed Shelf Life	36 Month
Stability Quantity	35Vials

Sr. NO.	TESTS	SPECIFICATION	REFERENCE
1.	**Description:**		
	Before reconstitution	White or off white lyophilized cake filled in clear colorless glass vial USP type I.	In House
	After reconstitution	Clear colourless solution is produced after reconstitution with 10ml water for injection.	In House
2.	**Identification**		
	A. By IR	The infrared absorption spectrum of the filtrate is concordant with the reference spectrum of Cyclophosphamide.	BP Monograph
3.	**Acidity (pH)**	Between 3.0 to 6.0	BP Monograph
4.	**Bacterial Endotoxins Test**	NMT 0.0625 EU/mg of Cyclophosphamide.	BP Monograph (Appendix XIVC)
5.	**Sterility (By Membrane Filtration)**	Sterile	BP Monograph (Appendix XVI A)
6.	**Particulate Matter.**		
	Visible Particle	It should be free from any type of visual particles	BP Monograph (Appendix XIII A)
	Sub visible particles, By Light Obscuration Particle Count Test.	For 10 µm to less than 25 µm - NMT 6000 particles For 25 µm or greater - 600 particles	

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21/06/23	21/06/23	21/06/23	

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STABILITY PRODUCT SPECIFICATION

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PRODUCT CODE	CY/006	**EFFECTIVE DATE**	21/06/23
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SPECIFICATION NO.	KPL/SPC/SI/104-00	**VERSION NO.**	00
STP NO.	KPL/STP/IN/104-00	**SUPERSEDES NO.**	Nil
Page No.	2 of 2		

Sr. NO.	TESTS	SPECIFICATION			REFERENCE
7.	**Related substances: (By TLC)**	Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) (1%)			BP Monograph
8.	**Assay: Each vial contain: (By Titration)**				
	Active ingredient	**Label Claim**	**Limit**		BP Monograph
	Cyclophosphamide (Anhydrous) BP	200 mg	92.5% to 107.5% (185.0 mg to 215.0 mg)		

REVISION HISTORY:

Sr. No.	Change control No.	Revision No.	Reason for Revision
01	CC/23/06/QC/039	00	New Specification

END OF DOCUMENT

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QUALITY CONTROL DEPARTMENT

PRODUCT STANDARD TEST PROCEDURE

PRODUCT NAME	**CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/VIAL**		
GENRIC NAME	CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/ VIAL		
PRODUCT CODE	CY/006	**EFFECTIVE DATE**	21/06/23
GRADE	BP-2022	**REVIEW DATE**	20/06/25
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SPECIFICATION NO.	KPL/SPC/IN/105-00	**SUPERSEDES NO.**	Nil
PAGE NO.	1 of 14		

1. DESCRIPTION:

Before reconstitution:

Procedure: Take the sample and check visually.

Observation: White or off white lyophilized cake filled in clear colorless glass vial USP type I.

After reconstitution:

Procedure: Take one vial and remove the flip off seal, add 10ml water for injection with the help of syringe and gently swirl the vial until the content is dissolved.

Observation: Clear colourless solution is produced after reconstitution with 10 ml water for injection.

2. IDENTIFICATION:

A. BY IR:

Sample preparation: Shake a containing the equivalent of 0.2g of anhydrous cyclophospahmide dilute with 2 ml chloroform, and filter. Use the filtrate as sample in infrared Spectrum test.

Procedure: Before start analysis of reference/working standard and sample perform background and then start analysis. Place reference/working standard on ATR and record the chromatogram at 650 cm^{-1} to 4000 cm^{-1}. Similar procedure follows for prepared sample.

Acceptance Criteria: The infrared absorption spectrum of the filtrate is concordant with the reference spectrum of Cyclophosphamide.

B. BY Chemically:

Silver Nitrate Solution: Dissolve 5g of silver nitrate in 100ml of water

Sample preparation: Extract a quantity containing the equivalent of 0.1g of anhydrous Cyclophosphamide with ether and evaporate the extract dryness.

Procedure: Dissolve the residue in 10 ml of water and add 5ml of silver nitrate solution: no precipitate is produced. Boil.

Acceptance criteria: A white precipitate is produced which is insoluble in nitric acid but soluble in 5 M ammonia from which it is reprecipitated on the addition of nitric acid.

3. Acidity (pH):

Procedure: Select one sample vial and reconstitute with 10 ml of water for injection and dissolve the content completely by shaking and transfer the content to a beaker and measure the pH of the solution.

Acceptance criteria: Between 3.0 to 6.0.

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4. UNIFORMITY OF WEIGHT:

Procedure: Select 20 sample vials and remove the printed label of the vial and flip off seal and then take the filled weigh of the vial (W1). Remove the content of vial and rinse two to three times with water and then with alcohol, and place the vial and stopper in the oven for drying and after drying take the vial and stopper from oven and place in the dessicator to attain room temperature and weight again (W2)

$$\text{Average mass} = \frac{\text{Average weight of filled vials} - \text{Average weight of empty vials}}{10}$$

Acceptance criteria: 415mg ±10% (373.5 mg to 456.5 mg).
(Not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation i.e. 10% and none deviates by more than twice that percentage).

5. UNIFORMITY OF CONTENT: (Method A)

Preparation of 0.1M Silver Nitrate

Dissolve 17.0gm of silver nitrate in sufficient water to produced1000 ml Standardize the solution in the following manner.

STANDARDIZATION:

Perform the standardization in triplicate and calculate the % RSD should not more than 0.2%.
Weigh accurately about 0.150 gm of sodium chloride, (previously dried at about 110^0cfor 2 hours). Dissolve it in 5ml of water. Add 5 ml of acetic acid, 50 ml of methanol and 0.15ml of eosin solution. stir, preferably with magnetic stirrer, and titrate with 0.1M silver nitrate solution.
Each 1ml of 0.1M silver nitrate is equivalent to 0.005844 gm Nacl.
Calculation;

$$\text{Molarity} = \frac{\text{Wt. of sodium chloride (mg) x Potency of Nacl x 0.1}}{\text{Volume consumed (ml) x 5.844 x 100}}$$

Preparation of ammonium iron (III) sulfate solution: Taken 10gm of ferric ammonium sulfate dilute 100 ml water. If necessary filter before use.

Preparation of 0.1M Ammonium Thiocyanate

Weight accurately 7.612gm of ammonium thiocyanate in a 1000ml volumetric flask and add sufficient water to dissolve properly and dilute to 1000ml with water. Standardize the solution in the following manner.

STANDARISATION (TRIPLICATE):

Pipette 30ml of 0.1 M silver nitrate standard solution in a glass stoppered flask and titrate with water to 50ml, add 2 ml of nitric acid 2 ml of ferric ammonium sulphate solution and titrate with to 0.1M ammonium thiocyanate solution to the first appearance of a red-brown colour.

Calculation;

$$\text{Molarity} = \frac{\text{Volume taken of 0.1M silver nitrate (ml) X Molarity of 0.1M silver nitrate}}{\text{Volume consumed (ml) of 0.1M ammonium thiocyanate}}$$

Preparation ferric ammonium sulfate: Taken 10 gm of ferric ammonium sulfate dilute 100 ml water.

Procedure: Taken 1 vial of anhydrous Cyclophosphamide in 300 ml of chloroform, shake vigorously for 15 minute, filter (whatman GF/F is suitable) and wash the filter with 15 ml of chloroform. Evaporate the combined filtrate and washing to dryness and dissolve the residue in 50 ml of a 0.1% w/v solution of sodium hydroxide in ethane-1,2-diol under a reflux condenser for 30 minutes and allow to cool. Rinse the condenser with 25 ml of water, add 75 ml of propan -2-ol, 15 ml of 2M nitric acid, 10 ml of 0.1M silver nitrate and 2 ml of ammonium iron (III) sulfate solution and titrate with 0.1 M ammonium thiocyanate volumetric solution. Each ml of 0.1 M silver nitrate volumetric solution is equivalent to 13.05 mg of cyclophospahmide. Calculate the content cyclophospahmide in the sealed container.

Repeat the procedure with a further nine sealed containers.

Calculation:

Calculate the average content of assay % of 10 results. Then calculate minimum % and maximum % from the 10 results against the average content.

Acceptance Criteria: 85% to 115% of average content.

(**NOTE:-** The preparation complies with me test if each individual content is between 85 percent and 115 percent of the average content. The preparation fails to comply with the test if more than one individual content is outside these limits or if one individual content is outside the limits of 75 percent to' 125 percent of the average content.

If one individual content is outside the limits of 85 percent to 115 percent but within the limits of 75 percent to 125 percent) determine the individual contents of another 20 dosage units taken at random. The preparation complies with the test if not more than one of the individual contents of the 30 units is outside 85 percent to 115 percent of the average content and none is outside the limits of 75 percent to 125 percent of the average content.)

6. STERILITY ANALYSIS

EQUIPMENT REQUIRED:

Laminar air flow

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21/06/23	21/06/23	21/06/23	

PRODUCT NAME	**CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/VIAL**		
GENRIC NAME	CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/ VIAL		
PRODUCT CODE	CY/006	**EFFECTIVE DATE**	21/06/23
GRADE	BP-2022	**REVIEW DATE**	20/06/25
STP NO.	KPL/STP/IN/105-00	**VERSION NO.**	00
SPECIFICATION NO.	KPL/SPC/IN/105-00	**SUPERSEDES NO.**	Nil
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Sterile filtration manifold assembly
Bio-safety cabinet
Vacuum pump
Manifold assembly

Membrane Filtration apparatus required:

Sterile Filtration Cups
Sterile filtration flask
Sterile Scissors
Sterile Forceps
Suction flask
Suction tube
Vial opener

Material required:

Sterile 0.45 µm cellulose nitrate membrane filter paper 47 mm diameter
70% filtered IPA solution
Sterile lint free cloth

Media required:

Sterile Fluid Thioglycollate Medium (FTGM 100 ml)
Sterile Soybean Casein Digest Medium (SCDM 100 ml)
Sterile 0.1% peptone water

PROCEDURE

Media Preparation and Sterilization

Prepare and sterilize the media mentioned above as per SOP No. MB005, "Receipt, storage, preparation, usage and sterilization of media".

Sterilization of Glassware and Accessories:

All the glassware & Accessories used in the Sterility Test shall be sterilized in HPHV Steam Sterilizer as per SOP No. MB083, "Operation, cleaning and maintenance of HPHV Steam Sterilizer".

Sample Quantity for analysis:

Draw 20 numbers of sample containers from each batch / lot for Sterility analysis.

Sterility by Open System (Membrane Filtration Method)

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Collect the 20 vials of Cyclophosphamide for injection USP 500 mg/ vial, which are to be tested for sterility in clean SS trays. Wipe each vial individually with 70% filtered IPA solution. Place the samples in clean SS trays and keep it in the dynamic pass box. Enter the sterility area as per SOP (SOP No.:MB088). Clean the LAF bench with 70% filtered IPA and switch on the UV light for not less than 15 minutes. After 15 minutes switch off UV light of LAF. Ensure that the pressure differential is between 10-15 mm of water scale. Take out the samples vials from the Dynamic Pass Box and again sanitize the vials with filtered 70 % IPA and transfer the sample vials under LAF. Allow the vials to dry. Before starting the sterility analysis, expose the SCDA plates under LAF and sterility room at specified locations. Arrange the samples and other accessories on LAF bench. Aseptically connect the filtration assembly under LAF. Now place 0.45µ sterile membrane filters between filtration cup and receptacle with the help of sterile forceps. Now switch on the vacuum pump.

Test sample:

Pre-wet the membrane filter with approximate 50 ml of sterilized 0.1 % peptone water and filter the fluid by employing vacuum to facilitate the filtration. Take 20 numbers of vials and reconstitute each vial with 25 ml of sterile water for injection. Mix the solution properly and then filter the half of the content of the sample from each vial through the filtration assembly by employing vacuum. Rinse the membrane filter paper with 3×100 ml of 0.1% sterile peptone water (3 times rinsing with 100 ml). Aseptically open the filtration assembly and hold the membrane filter with sterile forceps. Then cut the filter paper into two equal halves with the help of sterile scissor. Put one half of the membrane filter into FTGM tube and another half in SCDM tubes. After completion of work transfer all inoculated media tubes into incubation room through dynamic pass box. Incubate the FTGM tubes at 30-35° C and SCDM tubes at 20-25° C for 14 days.

Negative Diluting Fluid control:

Filter 100 ml of 0.1% peptone water through 0.45 µm membrane filter paper instead of product. Cut the Membrane filter paper into two equal halves with sterile scissor and transfer one half to FTGM and another half to SCDM and label as negative control. The negative control shall be incubated parallel with product. The negative control shall be performed in the last of the testing activity. The tubes of negative control having SCDM and FTGM shall be incubated in same way as for product to check the integrity of system. Incubate the FTGM tubes at 30-35° C and SCDM tubes at 20-25° C for 14 days.

Positive control:

Separately carry out positive control test under bio-safety cabinet of MLT room. Start the Bio-safety cabinet as per SOP No. MB105, "Operation, cleaning and maintenance of Bio-safety Cabinet" and prepare one positive

control tube by inoculating aseptically 10-100 CFU in FTGM tubes with *S. aureus* (ATCC 6538), *P. aeruginosa* (ATCC 9027), *Clostridium sporogenes* (ATCC 19404). Similarly prepare SCDM positive controls by inoculating 10-100 CFU with *Candida albicans* (ATCC 10231), *Aspergillus brasiliensis* (ATCC 16404), *Bacillus spizizenii* (ATCC 6633). Incubate FTGM positive control tube at 30-35° C for NMT 3 days and SCDM tube at 20-25° C for NMT 5 days.

Observations And Interpretation of Results:

Observe the sample under test, for microbial growth visually on daily basis up to the end of incubation period. Record all the observations as per respective format No. MB050/F02, "Sterility test report".

If no evidence of microbial growth is observed then the sample passes the test for sterility. Visually examine the media tube daily to its conclusion for macroscopic evidence of Microbial growth.

If material is being tested turn into the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1ml) of the medium to fresh tubes of the same medium, and then incubate the original and transfer tubes for not less than 4 days.

If no evidence of microbial growth is found in sample, the product to be examined complies with the test for sterility. Destruct all the positive controls after confirmation of growth in both SCDM and FTGM tubes as per SOP No. MB062.

If there is evidence of microbial growth (confirmed microscopically), reserve the containers & then follow SOP No. MB061, "Out of specification results in Microbiology lab"

- If no growth is found in the retest then the sample passes the test for sterility.
- If microbial growth is observed and confirmed microscopically in the retest, the sample fails the tests for Sterility.
- **Limit:** Should be sterile

7. PARTICULATE MATTER:

8.1 Visible particle:

Collect 10 vials of Cyclophosphamide for injection USP 500 mg/ vial and reconstitute each vials with 25 ml of water for injection and mix the solution properly to form a clear solution. Check each vial vertically and horizontally against the black and white panel with illuminated light for the presence of visible particle. Observe the vial content for about 5 seconds in front of white panel followed by its observation in front of

black panel of visual inspection board. Check the vials for visible particle and note down the results as per format no.: MB096/F03, "Visual particulate matter test report" for presence of Black particles (BP), White particles (WP), Fiber (F), Glass Pieces (GP), and Other Particles (OP) if any.

Acceptance Criteria: it should be free from any type of visual particles.

8.2 **Light obscuration particle count test.**

Environment Test: Environment test shall be performed prior to analyze the samples. Take cleaned 100ml screw cap bottle Perform analysis for particulate matter test by LPC as per SOP No. MB096." Operation and calibration of Liquid Particle Count".

Acceptance criteria: NMT 25 particles of ≥ 10µm per 25mL.

Test Procedure: After visual test same sample vials shall be used for sub-visible particle count by LPC. Transfer the content of each vial into a cleaned particle free container. Before performing the test by LPC ensure that there is no air bubble present in the sample solution which is to be tested, if air bubble is entrapped in the test sample solution allow it to stand for more than 2 min. Determine the particle count of ≥10µm particles and ≥25µm from all the 4 consecutive runs each of 05 ml from sample container. Disregard the results obtained for the first portion & calculate the mean number of particle counts of last three runs. The obtained mean particle count is the result per container. Take the print outs of the test report.

Acceptance criteria: The preparation complies with the test if the average number of particles present in the unit tested does not exceed 6000 per container equal to or greater than 10µm and does not exceed 600 per container equal to or greater than 25µm.

8. BACTERIAL ENDOTOXIN TEST:

Gel Clot Method is used for the analysis of Cyclophosphamide for injection USP 500 mg/ vial. In gel clot technique, equal volume of LAL & diluted test specimen are mixed in tubes and incubated at 37±1°C for 60 ± 2 min. The tubes are then observed for gel clot formation.

A positive response in the gel clot test indicates the Endotoxin in the sample, which equals or exceeds the reagent labelled sensitivity represented by the symbol lambda (λ).

Requirements:

Depyrogenate all glassware and other heat-stable materials in a Dry heat sterilizer.

LAL reagent (Label claim should be confirmed)

CSE (Control Standard Endotoxin)

LRW (Lal Reagent Water)

Vortex Mixer
Endotoxin free reaction tubes (10 X 75 mm)
Endotoxin free dilution tube (13 x 100 mm)
Heating block
Micropipette
Endotoxin-free disposable micropipette tips
Gloves
Stopwatch

Procedure:

Preparation of CSE dilution:

Remove Aluminium seal of the CSE vial without opening the rubber stopper. Carefully remove the stopper. Keep the stopper in a clean surface without touching the inner portion of stopper. Reconstitute the lyophilized CSE with LRW as per manufacturer's recommendation to obtain particular concentration of endotoxin.

Immediately after reconstitution, vortex and mix intermittently for 02 minutes as per mfg's CoA. Vortex and mix vigorously for at least 1 minute prior to each use. Further dilution of CSE depends on the endotoxin content /ml after reconstitution. Dilute the CSE in the reconstituted vial so as to get λ (λ is sensitivity of LAL). Do not use CSE after 28 days of reconstitution or as mentioned in the supplier's CoA.

For e.g.

For CSE preparation up to λ value (Equivalent to lysate sensitivity)

If we receive the CSE with the sensitivity of 4400 EU/vial against a particular lysate (as per COA of supplier), reconstitute it with 4.4 ml LRW as per vendor recommendation to get 1000 EU/ml concentration and serially diluted as follows to get equivalent to λ (when λ is 0.125). (Refer Table 1 for dilution).

For CSE preparation equivalent to λ

Table-1
Dilution of CSE

Dilution No.	CSE Preparation	LRW Quantity (ml)	Concentration (EU/ml)
1.	One CSE vial	4.4	1000
2.	0.1 ml of 1000 EU/ml	0.9	100

Dilution No.	CSE Preparation	LRW Quantity (ml)	Concentration (EU/ml)
3.	0.1 ml of 100 EU/ ml	0.9	10
4.	0.1 ml of 10 EU/ ml	0.9	1
5.	0.5 ml of 1.0 EU/ ml	0.5	0.5 (4λ)
6.	0.5ml of 0.5EU/ ml	0.5	0.25
7.	0.5ml of 0.25 EU/ ml	0.5	0.125

Note: - CSE of 4λ is used in the analysis.

CSE Storage:

Lyophilized CSE should be stored at 2-8°C and can be used up to the expiry date. Reconstituted CSE is to be stored in a refrigerator at 2-8°C for intermittent use for up to 28 days or as per the supplier's COA.

Reconstitution of LAL reagent:

Remove Aluminium seal of the Lysate vial without opening the rubber stopper. Tap on the rubber stopper and on side walls of the vial to collect the LAL powder in the bottom of the vial. Carefully remove the stopper. Keep the stopper in a clean surface without touching the inner portion of the stopper. Reconstitute the lyophilized LAL reagent with LRW as per the COA. Stop the re-constituted vial. Do not shake and vortex, mix gently avoiding formation of air bubbles and keep aside till clear solution is not visible.

LAL reagent Storage:

Lyophilized LAL reagent should be stored at 2-8°C. Reconstituted LAL reagent ideally should be stored in a refrigerator at 2-8°C during intermittent use for up to 24 hours

Preparation of sample dilution:

- Take two vials of Cyclophosphamide for injection USP 500 mg/ vial and reconstitute each vial with 25 ml of sterile water for injection or LRW. After reconstitution, make a pool and transfer into depyrogenated beaker (SOLUTION A).
- Calculate the MVD of the production by following formula:

$$MVD = \frac{EL \times conc.\ [mg/ml]}{\lambda}$$

- Endotoxin Limit (EL) = NMT 0.0625 EU/mg of Cyclophosphamide.

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GENRIC NAME	CYCLOPHOSPHAMIDE FOR INJECTION BP 200 MG/ VIAL		
PRODUCT CODE	CY/006	**EFFECTIVE DATE**	21/06/23
GRADE	BP-2022	**REVIEW DATE**	20/06/25
STP NO.	KPL/STP/IN/105-00	**VERSION NO.**	00
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- Lysate sensitivity (λ) of the Lysate used= 0.125 EU/ML
- Potency : 20 mg/ml

1. $MVD = \frac{EL \times conc.\ [mg/ml]}{\lambda} = \frac{0.0625\ EU/mg \times 20\ mg/ml}{0.125} = 10$

2. MVD/4 = 2.5

Dilution:

Take 1.0 ml sample from solution A and add 1.5 ml LRW (Solution B)

Use solution E for further analysis.

Note: Product shall be tested at MVD/4.

Testing Procedure:

Performed the Bacterial Endotoxin test for products by gel clot techniques as per below:

Negative Product Control (NPC):

- Label 2 endotoxin free reaction tubes having 10mm×75 mm dimensions (for each sample) as NPC, along with sample identification.
- Add 50 µl of sample solution from solution B and 50µl of LRW in to each of 2 reaction tubes.

Positive Product Control (PPC):

- Label 2 endotoxin free reaction tubesas PPC, along with sample identification.
- Add 50µl of sample solution from dilution B and 50 µl of CSE 0.5 EU/ML (equivalent to 4λ) in to each of 2 reaction tubes.

Negative Water Control (NWC):

- Label 2 endotoxin free reaction tubes as NWC. Add 100 µl of LRW into each of 2 reaction tubes.

Positive Water Control (PWC):

- Label 2 endotoxin free reaction tubes as PWC. Add 50 µl of CSE 0.5 EU/ML solution into each of 2 reaction tubes then add 50 µl LRW.

  Note: One set of NWC and PWC are enough for all the samples tested at once. But mention the results of these tests in all the individual sample reports.

Addition of LAL reagent:

- Add 100 µl of reconstituted LAL reagent to all the reaction tubes quickly in the end, starting from NWC and then NPC, PWC and PPC respectively.

Incubation:

- Mix the solution and lysate gently and place in the Heating block at 37 ± 1 °C for 60 ± 2 minutes. Record the time at which tubes are placed in the heating block.

Observation:

- After incubation, take each tube from the heating block and invert it through about 180° in one smooth motion. If a firm Gel has formed that remain in place upon inversion, record the results as Positive (+Ve).
- A negative result is characterized by the absence of firm gel or by formation of a viscous gel that does not maintain its integrity. Record the result as negative (-ve). The sample being examined passes the test.
- Handle the tubes with care to avoid subjecting them to unwanted vibration, so that the gel formed remain intact.

Note:- Record the results in respective data sheet.

Acceptance Criteria:

The sample under test complies with the test for Bacterial Endotoxins, when a Negative result is found in both tubes of NPC. The sample under test doesn't comply with the test for Bacterial Endotoxins, when a Positive result is found in both tubes of NPC.

The test is invalid in the following conditions:

- One or both the tubes of PWC shows Negative result
- One or both the tubes of NWC shows Positive result
- One or both the tubes of PPC shows Negative result
- One tube of NPC shows Positive result and the other is Negative results.

Repeat the test, if the test is invalid with a fresh set of reagents and materials which will have direct contact with the solutions. The sample complies with the test when a negative result is found in both tubes of NPC and the other controls are passed, in the repeat test.

Precautions:

- Do not freeze the Endotoxin solutions (CSE vial and dilutions), store at 2-8°C.

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- Remove freezed LAL reagent 15- 20 min before usage to attain room temperature.
- Do not vortex and or shake LAL Reagent. Mix gently without formation of air bubbles.
- Always use Endotoxin-free articles (like Tube, tips, micropipette, pipette) for the testing.
- Prepare the sample solutions by dissolving or diluting drugs. If necessary adjust the pH of solution to be examined (or dilution thereof) so that the pH of mixture of Lysate and sample solution falls within the pH range specified by the Lysate manufacturer, usually 6-8. The pH shall be adjusted by use of acid, base or a suitable buffer, as recommended by Lysate manufacturer. Acid and bases shall be prepared from concentrates or solid with water for BET in depyrogenated container.
- Put ON the heating block at least 15 – 20 min. prior so as to achieve the set temperature (37 ± 1° C).
- Every new batch of Lysate must undergo Lysate sensitivity confirmation test, before use.
- Avoid prolonged exposure of any BET reagent at room temperature as purified Endotoxin are unstable at room temperature in water which may lead to container absorption and molecular aggregation that makes the Endotoxin unavailable for LAL detection.
- Avoid inadequately controlled test parameters like accessories, reagents and analyst proficiency.

Acceptance Criteria: NMT 0.0625 EU/mg

9. RELATED SUBSTANCES: By TLC

Chromatography condition:

Use coating silica gel G.

Use the mobile phase as described below.

Apply 10 µL of each solution

Develop the plate to 15 cm

Procedure: After removal of the plate, Dry in air and heat at 100° for 10 minutes. Place the plate while hot in a chromatography tank in which is placed an evaporating dish containing equal volumes of a 5% w/v solution of potassium permanganate and hydrochloric acid. Close the tank and allow to stand for 2 minutes. Remove the plate and place it in a current of cold air until excess chlorine is removed and an area of coating below the line of application gives not more than a very faint blue color with potassium iodide and starch solution; avoid prolonged exposure to cold air. Spray the plate with potassium iodide and starch solution and allow to stand for 5 minute.

Mobile phase: Transfer 2 volumes of anhydrous formic acid, 4 volumes of acetone 12 volumes of water and 80 volumes of butan-2-one.

Test solution 1: Taken 1 vial of anhydrous cyclophosphamide and dilute with 10 ml ethanol (96%).

Test solution 2: Dilute 1 volume of test solution 1 to 100 volumes with ethanol (96%).
Limit: Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) (1%) .
Disregard any spot remaining on the line of application.

10. ASSAY: By Titration

Preparation of 0.1M Silver Nitrate

Dissolve 17.0gm of silver nitrate in sufficient water to produced1000 ml Standardize the solution in the following manner.

STANDARDIZATION:

Perform the standardization in triplicate and calculate the % RSD should not more than 0.2%.
Weigh accurately about 0.150 gm of sodium chloride, (previously dried at about 110^0cfor 2 hours). Dissolve it in 5ml of water. Add 5 ml of acetic acid, 50 ml of methanol and 0.15ml of eosin solution. stir, preferably with magnetic stirrer, and titrate with 0.1M silver nitrate solution.
Each 1ml of 0.1M silver nitrate is equivalent to 0.005844 gm Nacl.

Calculation;

$$\text{Molarity} = \frac{\text{Wt. of sodium chloride (mg) x Potency of Nacl x 0.1}}{\text{Volume consumed (ml) x 5.844 x 100}}$$

Preparation of ammonium iron (III) sulfate solution: Taken 10gm of ferric ammonium sulfate dilute 100 ml water. If necessary filter before use.

Preparation of 0.1M Ammonium Thiocyanate

Weight accurately 7.612gm of ammonium thiocyanate in a 1000ml volumetric flask and add sufficient water to dissolve properly and dilute to 1000ml with water. Standardize the solution in the following manner.

STANDARISATION (TRIPLICATE):

Pipette 30ml of 0.1 M silver nitrate standard solution in a glass stoppered flask and titrate with water to 50ml, add 2 ml of nitric acid 2 ml of ferric ammonium sulphate solution and titrate with to 0.1M ammonium thiocyanate solution to the first appearance of a red-brown colour.
Calculation;

$$\text{Molarity} = \frac{\text{Volume taken of 0.1M silver nitrate (ml) X Molarity of 0.1M silver nitrate}}{\text{Volume consumed (ml) of 0.1M ammonium thiocyanate}}$$

Preparation ferric ammonium sulfate: Taken 10 gm of ferric ammonium sulfate dilute 100 ml water.
Procedure: Taken 1 vial of anhydrous Cyclophosphamide reconstirute in 10 ml of water, taken 5ml equivalent to 100 mg of cyclophosphamide of sample in 30 ml of chloroform, shake vigorously for 15 minute, filters (whatman GF/F is suitable) and wash the filter with 15 ml of chloroform. Evaporate the combined filtrate and

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washing to dryness and dissolve the residue in 50 ml of a 0.1% w/v solution of sodium hydroxide in ethane-1,2-diol. Boil the solution under a reflux condenser for 30 minutes and allow to cool. Rinse the condenser with 25 ml of water, add 75 ml of propan -2-ol, 15 ml of 2M nitric acid, 10 ml of 0.1M silver nitrate and 2 ml of ammonium iron (III) sulfate solution R2 and titrate with 0.1 M ammonium thiocyanate volumetric solution. Each ml of 0.1 M silver nitrate volumetric solution is equivalent to 13.05 mg of cyclophospahmide. Calculate the content cyclophospahmide in the sealed container.

Repeat the procedure with a further nine sealed containers. Calculate the content of cyclophospahmide container from the average of the 10 individual results thus obtained.

REVISION HISTORY:

Sr. No.	Change Control No.	Revision No.	Reason for Revision
01	CC/23/06/QC/039	00	New STP

END OF DOCUMENT

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21/06/23	21/06/23	21.6.23	