Kanamycin Concentration Reduction During the VGXI Process

Background

Kanamycin is an aminoglycoside antibiotic which binds to prokaryotic ribosomes interfering with translation of mRNA. It is widely used as a selection mechanism during production of plasmid DNA for use in DNA vaccines and gene therapy products. Kanamycin is typically used during generation of master cell banks to isolate bacteria which have been transformed with plasmid containing kanamycin resistance genes. Kanamycin is also used in fermentation providing selective pressure ensuring bacteria maintain the plasmid containing kanamycin resistance. At VGXI, the removal of active kanamycin from final bulk product occurs during downstream processing. Downstream processing consists of cell lysis, solid/liquid separation of lysate by filtration, anion exchange membrane chromatography (AEX), hydrophobic interaction chromatography (HIC), Ultrafiltration (UF) and Diafiltration (DF) by tangential flow filtration (TFF), and aseptic filtration. See Table 1 for the estimated kanamycin concentration at each point during the process.

Table 1: Kanamycin Concentration Reduction during VGXI Process

Material	Process	Kanamycin Concentration (mg/ml)	Rationale
Master Cell Banks [1]	After cell banking	5.00E-02	Concentration of Kanamycin in the culture
Cell Paste [1]	After fermentation	5.00E-02	Concentration of Kanamycin in the culture
Resuspended Cells [2]	Before cell lysis	7.14E-03	7 fold dilution
Crude Lysate [3]	After cell lysis	1.79E-03	4 fold dilution
AEX Load [4]	Before AEX Capture	1.38E-03	1.3 fold dilution
AEX Eluate [5]	After AEX capture	1.38E-05	Estimated 100 fold concentration of plasmid with AEX bind and elute
HIC Load [6]	Before HIC Polishing	2.76E-06	5 fold dilution
HIC Eluate [7]	After HIC polishing	2.76E-08	Estimated 100x buffer exchange of eluate to load (10x wash, 10x elute)
UF Bulk, Washes [8]	After TFF buffer exchange	2.76E-12	>99.99% buffer exchange with continuous TFF of 10 DV

Expanded Rationale

1. ***Master Cell Banks and Cell Paste:***
   The kanamycin concentration in the cell bank and fermentation media is 50µg/mL, which is an over estimation of active Kanamycin. The kanamycin resistance gene codes for an enzyme, kanamycin kinase, which phosphorylates kanamycin, rendering it inactive. The actual amount of active kanamycin available at the end of fermentation cannot be generally quantified, as the degree of phosphorylation is dependent on fermentation length and fermentation cell density. Therefore, the starting kanamycin concentration provides a 'worst case' estimation of total active kanamycin in the bulk drug substance.

2. ***Resuspended Cells:***
   Once harvested, the cells are resuspended in resuspension solution at a ratio of 1:6. The initial step dilution of kanamycin is 7X.

3. ***Crude Lysate:***
   Cells are lysed by mixing resuspended cells with lysis solution at a 1:1 ratio (2X dilution). The lysed cell solution is neutralized with neutralization and precipitation solution at a 1:1 ratio to generate crude lysate (2X dilution). The total step dilution of kanamycin is 4X.

4. ***AEX Load:***
   Following filtration, the filtered lysate is diluted with purified water in a 3:1 ratio for AEX load. The total step dilution of kanamycin is 1.3X.

5. ***AEX Eluate:***
   Following the AEX load, the capsule is emptied and approximately 20 membrane volumes of solution are passed through the AEX membrane for wash. The wash volumes are then discarded. These steps will ensure removal of the majority of residual kanamycin in the AEX capsule. Approximately 9 membrane volumes are used for AEX elute and re-elute. Since this is not a direct dilution, the actual reduction in active kanamycin concentration is difficult to quantify. The 100 fold reduction is a conservative estimate based on total plasmid concentration increase following the AEX step. This will provide a 'worst case' estimation of total active kanamycin in the bulk drug substance.

6. ***HIC Load:***
   The AEX eluate is diluted with ammonium sulfate at a ratio of 1:4 for HIC load conditioning. The total step dilution of kanamycin is 5X.

7. ***HIC Eluate:***

   Following the HIC load, the equivalent of 3 column volumes, approximately 10 liquid volumes, are passed through the HIC column as wash and are not collected. Next, 3 column volumes, approximately 10 liquid volumes, are passed though the HIC column for the elute. Since this is not a direct dilution, the actual reduction in active kanamycin concentration is difficult to quantify. The 100 fold reduction is based on the total liquid volume added to the process during the HIC step. This will provide a 'worst case' estimation of total active kanamycin in the bulk drug substance.

8. ***UF Bulk, Washes:***

   10 diavolumes processed during continuous TFF would result in a 99.99% buffer exchange. This is used as an estimate for the final step in the plasmid purification process. All cGMP purification processes use ≥12 diavolumes in continuous TFF. The 10 diavolumes used for this calculation provide a 'worst case' estimation of total active kanamycin in the bulk drug substance.

Conclusion

In summary, the total active kanamycin in the bulk drug substance is less than 2.76fg/mL. This amount is undetectable by standard assay methods, typically at ppb (pg/mL) levels for kanamycin detection by LC/MS/MS.