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# 3.2.S.4.2 ANALYTICAL PROCEDURES

The analytical methods used for the release and stability testing of CX-024414, are described in the following section.

## 3.2.S.4.2.1 Appearance

CHVI-101557 (Lonza AG) are methods that are used to evaluate the appearance of samples (color, clarity, visible particulates) by visual inspection for cGMP release and stability testing of CX-024414 mRNA. SOP-0278 is performed in accordance with current USP <631>, EP 2.2.1, and EP 2.9.20 and CHVI-101557 is performed in accordance with current EP 2.2.1, EP 2.2.2 and EP 2.9.20.

**Procedure**

Product is assessed in a portable manual inspection hood consisting of an appropriate light source and vertical, non-glare white and matte black panel backgrounds. The light source is capable of maintaining an intensity of illumination, at the viewing point, between 2000 and 3750 lux. The product is observed against both black and white backgrounds under full-spectrum lighting. Opalescence of the product is assessed against a water control (Ultrapure or equivalent RNase free), if applicable. The product is examined for the presence of visible particulates. The results of the color, clarity, and visible particulates assessments are reported as required per the associated specifications, Section 3.2.S.4.1 {CX-024414}.

## 3.2.S.4.2.2 Identity (Reverse Transcription/Sanger Sequencing)

SOP-1019 is used to assess mRNA identity of CX-024414 by using RT-PCR (Reverse Transcription-Polymerase Chain Reaction) to create an amplified double-stranded cDNA product for Sanger sequencing on an ABI genetic analyzer. Sample electropherograms are then assembled and compared to the reference sequence to confirm the sequence of CX-024414. This method applies to the entire Open Reading Frame (ORF) of the product, from the start codon to the end of the triple stop codon. Microsynth performs identity testing for Lonza AG. The qualified Microsynth method aligns with the method validated by ModernaTX, Inc.

**Instrument, Equipment, and Reagents**

Instrumentation, equipment, and reagents for RT-PCR and Sanger Sequencing analysis are provided in Table 1. Standard laboratory equipment is not listed. Equivalent instruments and reagents may be substituted where indicated. Solutions prepared for use in this method are described in Table 2.

## Table 1: Instrument, Equipment, and Reagents

| Instrument and Equipment |
|---|
| Genetic Analyzer |
| Thermocycler |
| Thermomixer |
| Electrophoresis Device |
| Gel Imager |
| Biosafety Cabinets (BSC) |
| **Reagents** |
| CCI [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |
| [redacted] |

## Table 2: CX-024414 Primers

| **RT-PCR Primers** | |
|---|---|
| **Primer Name** | **5'-Sequence-3'** |
| CCI [redacted] | [redacted] |
| [redacted] | [redacted] |
| **Sequencing Primers** | |
| **Primer Name** | **5'-Sequence-3'** |
| CCI [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| [redacted] | [redacted] |

**Table 3: Solution Preparation**

| Solution | Composition |
|---|---|
| CCI | [redacted] |

## Sample and Control Preparation

The test samples and positive control are diluted to [redacted] C with CCI [redacted] into a microcentrifuge tube.

## Procedure

The test samples, a positive control, and negative control undergo an RT-PCR reaction using the RT-PCR master mix containing CCI [redacted]. Using a thermocycler, cDNA is made from the mRNA, and then PCR amplified using the following program settings (Table 4).

**Table 4: RT-PCR Thermocycler Program**

| Step | Temperature | Time |
|---|---|---|
| CCI | [redacted] | |

a) The typical CCI [redacted] For constructs longer than 3 kb (including CX-024414), lengthen the CCI [redacted] rounding up if needed.

The cDNA products of the RT-PCR samples and controls then undergo gel electrophoresis to confirm cDNA synthesis and amplification, and that the primers produced a band that is the expected size. The RT-PCR product samples and controls are purified using the CCI [redacted] clean-up kit. ExoSAP-IT reagent is added to the RT-PCR products and purified in a thermocycler using an ExoSAP thermocycling program (Table 5).

**Table 5: ExoSAP Thermocycler Program**

| Step | Temperature | Time |
|---|---|---|
| CCI | [redacted] | |

The purified RT-PCR reactions and then pooled and undergo a Sanger Sequencing reaction. The samples and controls are mixed with the sequencing reaction master mix containing

CCI . The reaction mixtures of the samples and controls are plated into a PCR plate the corresponding sequencing primer is added per a predetermined plate map. Each sequencing reaction is prepared in triplicate. The plate is then loaded into a thermocycler and the sequencing thermocycling program (Table 6) is run.

**Table 6: Sequencing Thermocycler Program**

| Step | Temperature | Time |
|---|---|---|
| CCI | | |

The sequencing reactions are then purified using the CCI solution is added to each reaction well of the sequencing plate and is then mixed at CCI using a thermomixer. The purified sequencing reactions are loaded into the genetic analyzer and are run using the CCI assay (instrument protocol: CCI ).

**Data Analysis and Reporting**

Utilizing the SeqScape software, the sequencing data of the samples and positive control are assembled and compared to their respective reference sequence.

**System Suitability and Test Article Acceptance Criteria**

System suitability and acceptance criteria are summarized in Table 7.

**Table 7: System Suitability and Test Article Acceptance Criteria**

| Category | Parameter | Acceptance Criteria |
|---|---|---|
| System suitability | RT-PCR reaction success and specificity. | CCI |
| System suitability | No Template Control | |
| System suitability | Sequencing Positive Control | |
| Sample Suitability | Sample Sequencing Coverage | |
| Sample acceptance | Sample Identity | Evaluate the alignment of the consensus sequence to the reference sequence. If the consensus sequence matches the reference sequence with 100% homology, the test article nucleotide sequence conforms to the identity test specification. |

### 3.2.S.4.2.3 Total RNA Content (UV)

GROUP-107939 (Lonza AG), mRNA Concentration by NaOH Digest, are digest methods used in the determination of CX-024414 mRNA concentration by UV absorbance on a plate reader. Beer's Law is used to calculate the concentration.

**Instrument, Equipment, and Reagents**

Instrumentation, equipment, and reagents are provided in Table 8. Standard laboratory equipment is not listed. Equivalent instruments and reagents may be substituted where indicated. Solutions prepared for use in this method are described in Table 9.

**Table 8: Instrument, Equipment, and Reagents**

| Instrument and Equipment |
|---|
| Microplate Reader capable of measuring the absorbance of a sample in a microplate approved for UV measurement |
| Microplates with UV transparent flat bottom |
| Thermomixer C |
| RNase-free micro-centrifuge tubes |
| **Reagents** |
| CCI [redacted] |

**Table 9: Solution Preparation**

| Solution |
|---|
| CCI [redacted] |

**Sample and Standard Preparation**

CX-024414 samples in the concentration range of CCI [redacted] are diluted in CCI [redacted] at a CCI [redacted]. CX-024414 samples at a concentration CCI [redacted] are diluted in CCI [redacted] at a 1:10 ratio.

Sample digestion is prepared by combining CCI [redacted] of diluted sample.

Samples are prepared in triplicate, then each is digested in duplicate, for a total of 6 preparations.

Blank digestion is prepared by combining CCI [redacted], for a total of 6 preparations.

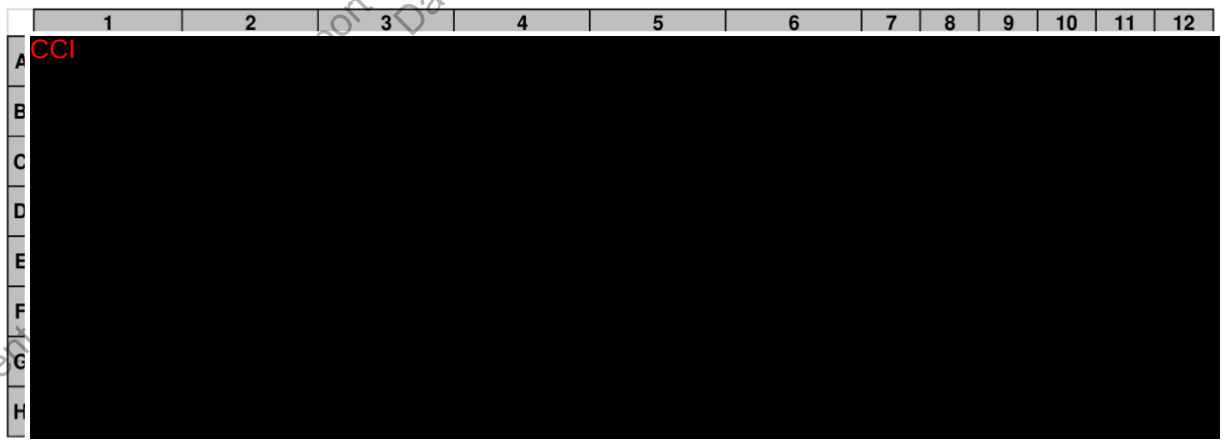
## Procedure

Vortex, centrifuge, and place blank and sample tubes on the ThermoMixer C, or equivalent, set to CCI. After CCI, remove blank and sample tubes and place on ice for CCI, then centrifuge. Add CCI to each blank and sample tube. Add CCI to each blank and sample tube. Vortex. Dispense CCI of each blank and sample into appropriate Microplate. See example plate map below (Figure 1). Perform Data Reduction as appropriate to blank the instrument. Read microplate according to protocol parameters below (Table 10).

**Table 10: Microplate Reader Protocol Parameters**

| Parameter | Condition |
|---|---|
| Read Mode | Absorbance |
| Read Type | Endpoint |
| Wavelength Settings | CCI |
| Plate Type Settings | |
| Read Area | Highlight all the wells to be read (wells contain blank and sample solutions) |
| Pathcheck settings | Check Pathcheck<br>• Water constant: should be filled automatically |
| Shake Settings | 5 seconds before first read |
| Speed Read | Leave blank |
| Under more settings | Check Calibrate box<br>• Carriage speed: Normal<br>• Read Order: Column |

**Figure 1: Example Plate Map**




The concentration of digested mRNA is calculated by the following equation:

$$mRNA\ Concentration\ \left(\frac{mg}{mL}\right) = \frac{\text{CCI}}{Pathlength}\ x\ SCC\ x\ Sample\ Dilution$$

Where,

CCI

SCC = Sequence Corrected Coefficient (obtain from product Summary of Analysis)
Pathlength = 1 cm

**System Suitability and Test Article Acceptance Criteria**

System suitability and acceptance criteria are summarized in Table 11.

**Table 11: System Suitability and Test Article Acceptance Criteria**

| Category | Parameter | Acceptance Criteria |
|---|---|---|
| System suitability | ΔOD of blank | ΔOD of blank well readings must be CCI |
| Sample acceptance | Concentration precision | CCI |
| Sample acceptance | ΔOD of sample | ΔOD of sample readings must be CCI |

## 3.2.S.4.2.4 Purity and Product-related Impurities (RP-HPLC)

GROUP-107927 (Lonza AG) are used to assess mRNA purity of CX-024414. The method separates mRNA species by size, using reverse phase ion-pair high performance liquid chromatography (RPIP-HPLC) and gradient elution. Detection is performed by UV at 260 nm. Total purity and impurities are calculated as percent peak area.

**Instrument, Equipment, and Reagents**

Instrumentation, equipment, and reagents for RPIP-HPLC analysis are provided in Table 12. Standard laboratory equipment is not listed. Equivalent instruments and reagents may be substituted where indicated. Solutions prepared for use in this method are described in Table 13.

**Table 12: Instrument, Equipment, and Reagents**

| Instrument and Equipment |
|---|
| HPLC system with UV detector, |
| Refrigerated Centrifuge |
| CCI |
| **Reagents** |
| CCI |

**Table 13: Solution Preparation**

| Solution | Composition |
|---|---|
| CCI | [redacted] |

**Sample and Standard Preparation**

Dilute CX-024414 to CCI in HPLC Grade water.

**HPLC Analysis and Operating Conditions**

Samples of CX-024414 are injected in duplicate, using a single diluted sample as described in sample preparation.

**Table 14: Example Injection Sequence**

| Sample Name | Number of Injections |
|---|---|
| Column conditioning (mRNA Reference Standards) | ≥ 5 |
| Water Blank | ≥ 5 |
| Sensitivity Solution | 1 |
| Reference standard | 5 |
| Water Blank | ≥ 1 |
| Sample 1, Prep 1 | 1 |
| Sample 1, Prep 2 | 1 |
| Sample 2, Prep 1 | 1 |
| Sample 2, Prep 2 | 1 |
| Samples 3 through 5 (2 preps each) | 1 |
| Water Blank | ≥ 1 |
| Bracketing reference standard (inject after every 5 samples and at the end of every sequence) | … |
| Water Blank | ≥ 1 |

**Table 15: HPLC Operating Conditions**

| Parameter | Condition |
|---|---|
| Mobile phase A (MPA) | CCI |
| Mobile phase B (MPB) | |
| Flow rate | |
| Column temperature | |
| Pre-Column Heater Temperature | |
| Post-Column Cooler | |
| Draw Speed | |
| Dispense Speed | |
| Needle Wash Settings | |
| Autosampler temperature | |
| UV detection wavelength | |
| Acquisition time | |
| Injection volume | |
| Gradient | **Time (minutes)** / **% MPA** / **% MPB** |
| | CCI |

**Data Analysis, System Suitability and Data Reporting**

Data Analysis

- Using the water blank, baseline subtract all chromatograms.
- Peaks are integrated and labeled for each reference standard and sample chromatogram as depicted in the representative chromatographic profiles (Figure 2)
- CX-024414 Profiles will have 3 peaks areas identified and integrated: Pre-Main Peak, Main Peak, Post-Main Peak.
- The signal-to-noise ratio of the main peak in the detectability standard injection is calculated using the USP S/N calculation.
- Calculate the % carryover by comparing the peak areas in the blank prior to the sensitivity standard and all carryover blank injections to the average total peak area of the five initial standard injections.
- The percent recovery of the main peak area for each bracketing standard is calculated with respect to the average main peak area from the five initial reference standard injections.

- The percent agreement of the main peak retention time for each bracketing standard is calculated with respect to the average main peak retention time from the first five reference standard injections.
- The % relative standard deviation (% RSD) of the main peak area and retention time for the five initial standard injections will be calculated.
- The relative percent peak area of the mRNA peak, or percent purity, is calculated as follows:

$$\%\ Purity = \frac{Peak\ area\ of\ mRNA\ peak}{Total\ chromatographic\ peak\ area} \times 100\%$$

- The relative percent peak area of the total impurities is calculated as follows:

$$\%\ Total\ Impurities = \frac{Total\ peak\ area\ of\ impurities}{Total\ chromatographic\ peak\ area} \times 100\%$$

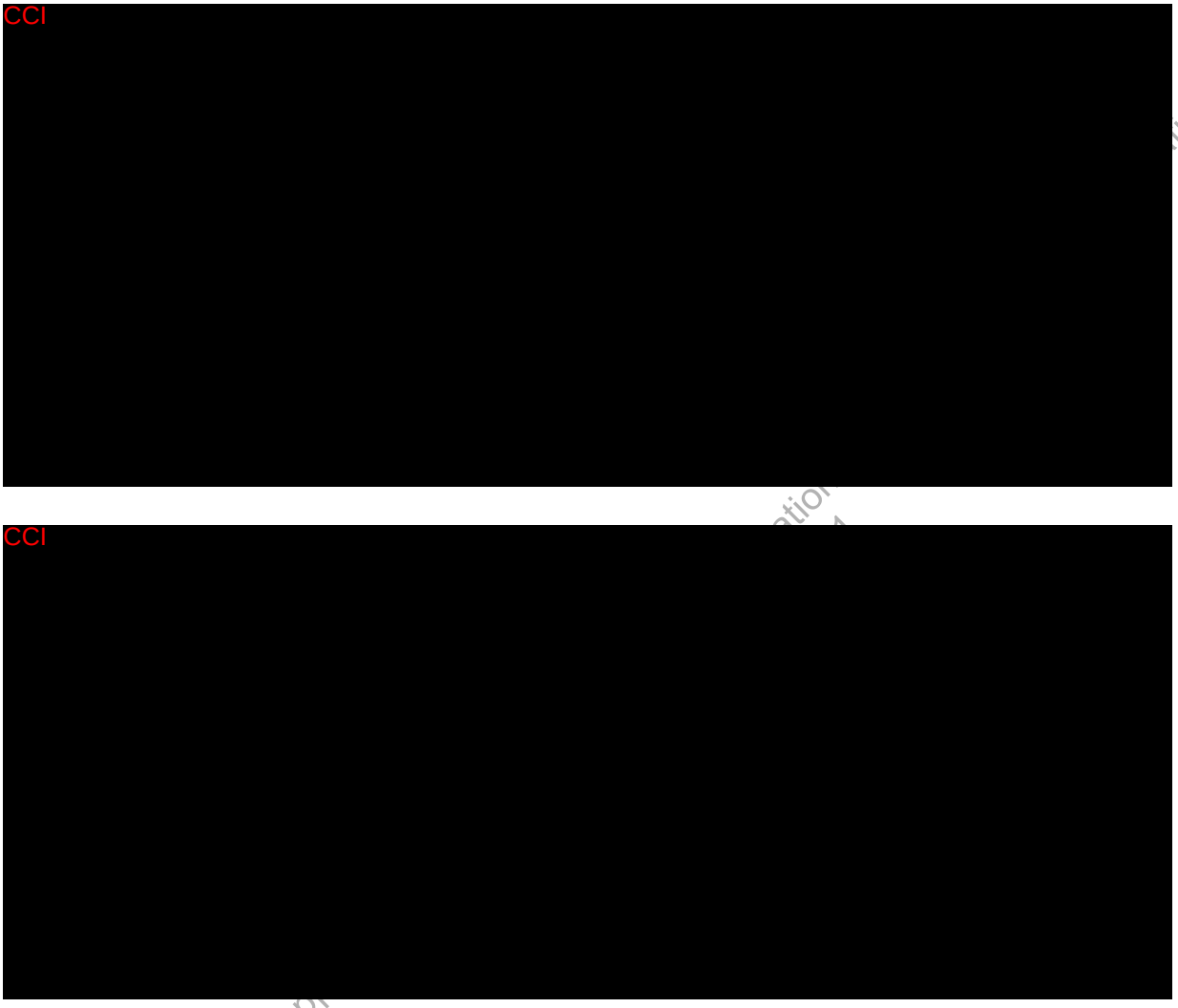
- The total peak area is the sum of the peaks noted above, not to include artifact and diluent peaks.
- Calculate the Absolute Difference between the main peak % area in duplicate injections of each sample.
- Calculate the percent recovery of the total peak area for each sample replicate with respect to the average total peak area from the first five reference standard injections.

**System Suitability and Test Article Acceptance Criteria**

**Table 16: System Suitability and Test Article Acceptance Criteria**

| Category | Parameter | Acceptance Criteria |
|---|---|---|
| System suitability | CCI | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| Sample acceptance | | |
| Sample acceptance | | |

**Figure 2: Representative Sample Chromatograms (Top Trace – Full Scale Drug Substance, Bottom Traces – Peak Detail, Drug Substance)**




## 3.2.S.4.2.5 % PolyA Tailed RNA and Tailless RNA (RP-HPLC)

GROUP-107934 (Lonza AG) are reverse phase high performance liquid chromatographic separation (RP-HPLC) methods which measure RNA containing a polyA tail. Separation in this method is highly influenced by the presence of a polyA tail; any RNA containing a polyA tail elutes in the main peak, whereas impurities (tailless mRNA and tailed mRNA variants) lacking a polyA tail have shorter retention times relative to the main peak. The method utilizes a gradient reverse phase high performance liquid chromatography separation with UV detection at 260 nm. Results are reported in relative percentages of total area.

## Instrument, Equipment, and Reagents

Instrumentation, equipment, and reagents for Tailed / Tailed Variant mRNA HPLC analysis are provided in Table 17. Standard laboratory equipment is not listed. Equivalent instruments and reagents may be substituted where indicated. Solutions prepared for use in this method are described in Table 18.

**Table 17: Instrument, Equipment, and Reagents**

| Instrument and Equipment |
|---|
| HPLC system with UV detector, CCI UHPLC System, or equivalent |
| CCI column, CCI |
| Refrigerated Micro-Tube Centrifuge, CCI , or equivalent |
| Analytical Balance, CCI , or equivalent |
| pH Meter, CCI or equivalent |
| **Reagents and Materials** |
| CCI |

**Table 18: Solution Preparation**

| Solution | Composition |
|---|---|
| CCI | |

## Sample and Standard Preparation

Reference standard and mRNA samples are diluted to CCI with HPLC grade water in a microcentrifuge tube and transferred to a low-volume HPLC Vial for analysis. mRNA samples are prepared in duplicate.

## Procedure

A single injection of sensitivity standard, and five replicate injections of reference standard, are performed at the beginning of each analysis on an HPLC system equipped with a CCI RP column. HPLC grade water is injected prior to the sensitivity solution, following the reference standard injections, and at the end of the sequence to assess diluent interference and carryover. Samples are prepared in duplicate and a single injection is performed for each replicate. Reference standard is injected to bracket every 3 samples (6 injections) and at the end of each analysis. Samples are stable in the autosampler, before injection, for up to 3 days. The chromatographic conditions for analysis are summarized in Table 19 and an example injection sequence in presented in Table 20. A representative chromatographic profile is shown in Figure 3.

**Table 19: HPLC Operating Parameters**

| Parameter | Condition |
|---|---|
| CCI | CCI |

| Time (minutes) | % MPA | % MPB |
|---|---|---|
| CCI | | |

**Table 20: Example Injection Sequence**

| Sample Name | Number of Injections |
|---|---|
| Column conditioning | ≥ 5 |
| Diluent (HPLC grade water) | ≥ 3 |
| Sensitivity standard | 1 |
| Reference standard | 5 |
| Diluent | ≥ 1 |
| Sample 1 Preparation 1 | 1 |
| Sample 1 Preparation 2 | 1 |
| Sample 2 Preparation 1 | 1 |
| Sample 2 Preparation 2 | 1 |
| Sample 3 Preparation 1 | 1 |
| Sample 3 Preparation 2 | 1 |
| Bracketing reference standard (inject after every 3 samples and at the end of every sequence) | 1 |
| Diluent | ≥ 1 |

## Data Analysis and Reporting

- The diluent injection immediately before the sensitivity standard is overlaid with any reference standard injection to verify there is no interfering peak present at the same retention time(s) of the peak(s) of interest.
- Peaks are integrated and labeled for each reference standard and sample chromatogram as depicted in the representative chromatographic profile (Figure 3).
- The signal-to-noise ratio of the main peak in the sensitivity standard injection is calculated.
- Resolution between the tailed variant and tailed mRNA peaks in the System Suitability Standard injections is calculated.
- % Relative Standard Deviation of the peak area and retention time of the tailed mRNA peak in the first five injections of reference standard is calculated.
- The percent recovery of the tailed mRNA peak area for each bracketing standard is calculated with respect to the average peak area from the first five reference standard injections as follows:

$$\%\,Recovery = \frac{Bracketing\ reference\ standard\ tailed\ mRNA\ peak\ area}{Average\ reference\ standard\ tailed\ mRNA\ peak\ area\ (n=5)} \times 100\%$$

- The percent agreement of the tailed mRNA peak retention time (RT) for each bracketing standard is calculated with respect to the average peak area from the first five reference standard injections as follows:

$$\%\,Recovery = \frac{Bracketing\ reference\ standard\ tailed\ mRNA\ peak\ RT}{Average\ reference\ standard\ tailed\ mRNA\ peak\ RT\ (n=5)} \times 100\%$$

- The percent recovery of the total mRNA peak area for each sample is calculated with respect to the average total peak area from the first five reference standard injections as follows:

$$\%\,Recovery = \frac{Total\ peak\ area\ of\ the\ sample\ replicate}{Average\ reference\ standard\ total\ peak\ area\ (n=5)} \times 100\%$$

- The relative percent peak area of the tailed mRNA peak, or percent purity, is calculated as follows:

$$\%\,Tailed = \frac{Peak\ area\ of\ tailed\ mRNA\ peak}{Total\ chromatographic\ peak\ area} \times 100\%$$

- The absolute difference of the tailed mRNA peak % area, or percent purity, is calculated as follows for each sample:

$$Absolute\ difference = Absolute\ value \left| Replicate\ 1\%\ tailed\ mRNA - Replicate\ 2\ \%\ tailed\ mRNA \right|$$

- The relative percent peak area of the total tailed variant peaks is calculated as follows:

$$\%\ Tailed\ Variants = \frac{Total\ peak\ area\ of\ tailed\ variant\ peak(s)}{Total\ chromatographic\ peak\ area} \times 100\%$$

- The mean percent Tailed and percent Tailed Variant mRNA for the duplicate sample injections are reported to one decimal place if CCI
- The mean percent Tailed and percent Tailed Variant mRNA for the duplicate sample injections are reported to one decimal place if greater than or equal to the quantitation limit of CCI

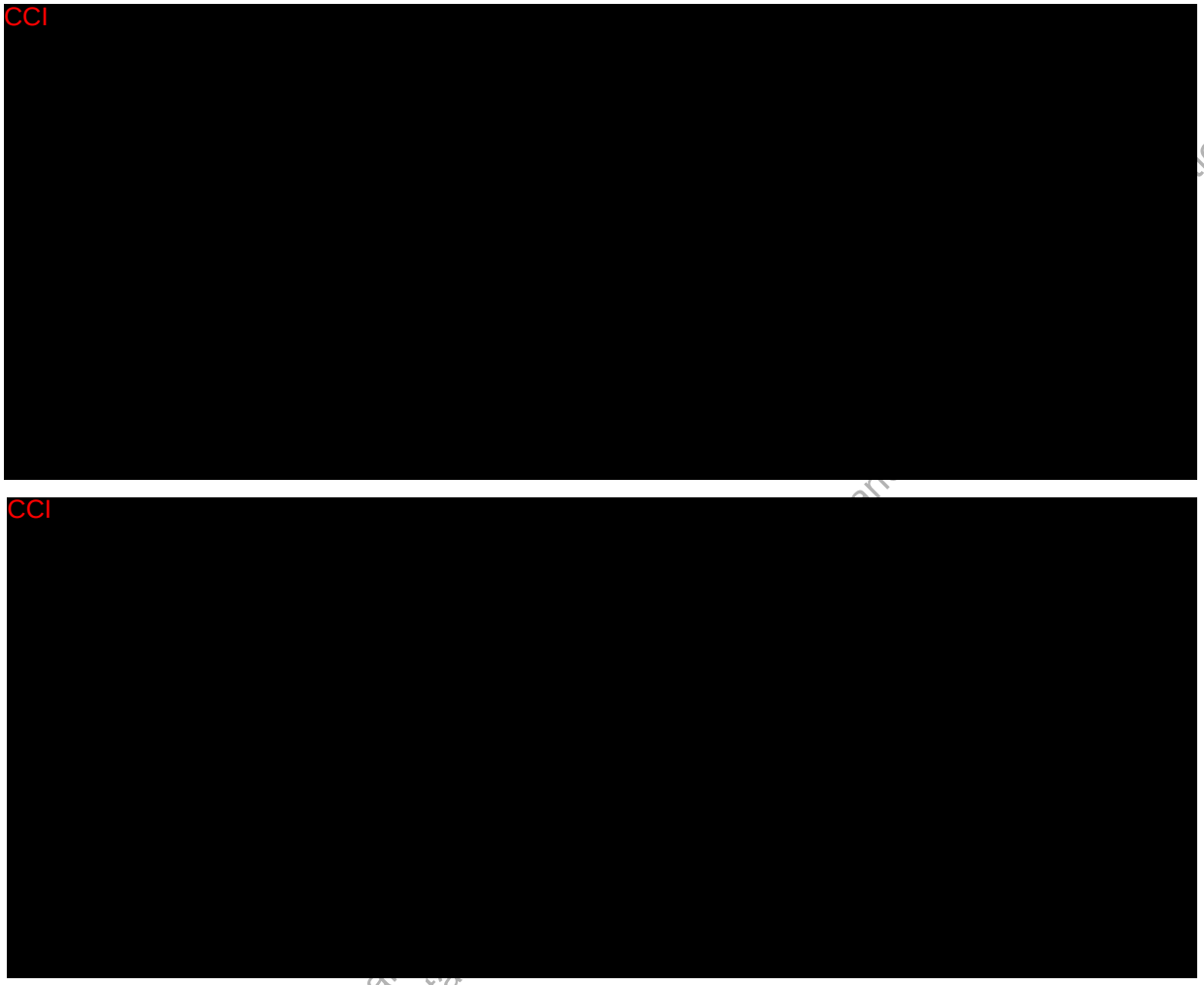
**System Suitability and Test Article Acceptance Criteria**

Reference standard is analyzed within each analysis to ensure the system is suitable for use on each day of analysis. System suitability and acceptance criteria are summarized in Table 21.

**Table 21: System Suitability and Test Article Acceptance Criteria**

| Category | Parameter | Acceptance Criteria |
|---|---|---|
| System suitability | CCI | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| Sample acceptance | | |
| Sample acceptance | | |

**Figure 3: Representative Sample Chromatogram (Top Trace – Full Scale, Bottom Trace – Expanded Scale)**




## 3.2.S.4.2.6 % 5' Capped (RP-UPLC-UV)

GROUP-107938 (Lonza AG) are used to assess mRNA integrity of drug substance by a length-based gradient using reverse phase ion-pair high performance liquid chromatography (RPIP-HPLC). Detection is performed by UV at 260 nm. The amount of each specie (capped and uncapped) is reported as a relative percentage of the total area.

**Instrument, Equipment, and Reagents**

Instrumentation, equipment, and reagents for RPIP-HPLC analysis are provided in Table 22. Standard laboratory equipment is not listed. Equivalent instruments and reagents may be substituted where indicated. Solutions prepared for use in this method are described in Table 23.

## Table 22: Instrument, Equipment, and Reagents

| Instrument and Equipment |
|---|
| HPLC system with UV detector |
| CCI column |
| CCI clear HPLC vial, CCI |
| **Reagents** |
| CCI |

## Table 23: Solution Preparation

| Solution |
|---|
| CCI |

**Sample and Standard Preparation**

Resolution marker and DS samples are diluted to CCI with HPLC grade water.

Sensitivity solution (reference standard) is diluted to CCI with HPLC grade water.

CCI mixture is added to a volume of the diluted marker/sample/sensitivity solution. Samples are incubated at CCI in preheated Thermomixer for CCI After incubation, samples are placed in a bead/ice bath for CCI solution, and CCI solution are then added to all samples. Samples are then added to HPLC vials for analysis.

**Procedure**

Blank injections are used to equilibrate the system/column then one injection of sensitivity solution followed by five replicate injections of Resolution marker solution; these are performed at the beginning of each analysis on an HPLC system equipped with an CCI column. Duplicate injections of each prepared sample are performed. Single diluent (HPLC grade water) injection and Reference standard are injected to bracket every 5 samples (10 injections) and at the end of each analysis. Samples are stable in the autosampler for up to 3 days. The chromatographic conditions for analysis are summarized in Table 24 and an example injection sequence in presented in Table 25. A representative chromatographic profile is shown in Figure 4

**Table 24: HPLC Operating Parameters**

| Parameter | Condition |
|---|---|
| CCI | |

**Table 25: Example Injection Sequence**

| Sample Name | Number of Injections |
|---|---|
| Column conditioning/Blank | 0 |
| Diluent Blank (HPLC grade water) | ≥ 2 |
| Sensitivity solution | 1 |
| Resolution Marker solution | 5 |
| Sample 1 prep 1 | 1 |
| Sample 1 prep 2 | 1 |
| Sample 2 prep 1 | 1 |
| Sample 2 prep 2 | 1 |
| Sample 3 prep 1 | 1 |
| Sample 3 prep 2 | 1 |
| Sample 4 prep 1 | 1 |
| Sample 4 prep 2 | 1 |
| Sample 5 prep 1 | 1 |
| Sample 5 prep 2 | 1 |
| Water blank | 1 |
| Resolution Marker Solution | 1 |
| Bracketing reference standard (inject after every 5 samples and at the end of every sequence) | 1 |
| Water blank | 1 |
| Wash step | Wash column with CCI [redacted] |

**Data Analysis and Reporting**

- The diluent/Blank injection immediately before the Resolution Marker solution is overlaid to verify there is no interfering peak present at the same retention time(s) of the peak(s) of interest.
- Peaks are integrated and labeled for each resolution marker and sample chromatogram as depicted in the representative sample chromatographic profile.
- The signal-to-noise ratio of the main peak in the sensitivity standard injection is calculated.
- Resolution between the Cap1 and Cap0 peaks are determined for all resolution marker injections.
- The % relative standard deviation (% RSD) of the Cap1 peak area and retention time for the first five resolution marker standard injections is calculated.
- The % relative standard deviation (% RSD) of the Cap1 peak retention time for all resolution marker standard injections is calculated.
- The percent recovery of the mRNA Cap 1 peak area for each bracketing standard is calculated with respect to the average Cap 1 peak area from the first five resolution marker injections as follows:

$$\%\text{Recovery} = \frac{A_{\text{bracket std}}}{\text{Avg}_{\text{std}}} * 100$$

where $A_{\text{bracket std}}$ = Cap1 peak area of bracketing standard

$\text{Avg}_{\text{std}}$ = average Cap1 peak area of first three marker injections (n=3

- The percent area of the components and Impurities, is calculated as follows:

$$\%\ Variants\ Area\ (\%\ Area) = \frac{Total\ peak\ area\ of\ all\ variants}{Total\ chromatographic\ peak\ area} \times 100\%$$

- Absolute Difference for Cap1 peak % area between duplicate sample preparations is calculated as follows:

$$Abs\ Difference\ between\ duplicate\ preps = \text{ABS(Prep 1 Cap 1 \% Area} - \text{Prep 2 Cap 1 \% Area)}$$

- The % Capped and % Cap1 is reported.

## System Suitability and Test Article Acceptance Criteria

Reference standard is analyzed within each analysis to ensure the system is suitable for use for each assay performed. System suitability and acceptance criteria are summarized in Table 26.

**Table 26: System Suitability and Test Article Acceptance Criteria**

| Category | Parameter | Acceptance Criteria |
|---|---|---|
| System suitability | CCI | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| System suitability | | |
| Sample acceptance | | |

**Figure 4: Representative Sample Chromatogram (Top Trace – Full Scale, Bottom Trace – Expanded Scale)**

CCI

CCI



### 3.2.S.4.2.7 Process-related Impurities (Residual DNA Template)

GROUP-107931 (Lonza AG) is used to detect and quantify the %w/w of residual plasmid present in CX-024414. The %w/w of residual plasmid is determined using real-time quantitative PCR (qPCR) designed to amplify the kanamycin resistance gene present in the parent plasmid of the mRNA product.

## Instrument, Equipment, and Reagents

Instrumentation, equipment, and reagents for qPCR analysis are provided in Table 27. Standard laboratory equipment is not listed. Equivalent instruments and reagents may be substituted where indicated. Solutions prepared for use in this method are described in Table 29.

**Table 27: Instrument, Equipment, and Reagents**

| Instrument and Equipment |
|---|
| Real-time PCR System |
| Biosafety Cabinets (BSC) |
| **Reagents** |
| CCI [redacted] |

**Table 28: qPCR Primers and Probes**

| Primer Use | Primer Name | 5'-Sequence-3' |
|---|---|---|
| CCI [redacted] | | |

**Table 29: Solution Preparation**

| Solution | Composition |
|---|---|
| CCI [redacted] | |

## Sample Preparation

The CX-024414 test sample is diluted CCI using Diluent 2. The CCI sample dilution is then serially diluted CCI using Diluent 3 until a CC sample dilution is obtained (6 total serial dilutions from the neat sample).

## Procedure

The qPCR master mix is prepared with CCI [redacted]. The qPCR master mix is then divided into 8-strip tubes where the sample, positive control, or negative control will be later added. Diluent 3 (the negative control) is added to the master mix tube designated as the NTC.

The test samples are prepared as described above. Each dilution of the test sample is then added to its corresponding master mix tube. The standard curve is prepared by initially diluting the Standard Linearized Plasmid to CCI [redacted] (Stock 1) with Diluent 2. The standard is then serially diluted from CCI [redacted] using Diluent 3. The standards containing CCI [redacted] are then added to its corresponding master mix tube.

The qPCR master mix with the samples, standards, and NTC are then plated in triplicate per a predetermined plate map. The plate is loaded onto a real-time PCR instrument and following program is run (Table 30).

**Table 30: Residual Plasmid Real-time PCR Program**

| Cycle | Temperature | Time |
|---|---|---|
| CCI [redacted] | | |

## Data Analysis and Reporting

An excel sheet is used to calculate the standard curve and sample concentrations. The copies/µL of each point of the standard curve and each sample dilution are calculated using the following equation and inputting the $C_T$, slope, and y-intercept of the standard curve.

$$\text{Copies}/\mu\text{L} = 10^{(Ct\ -\ y-intercept)/Slope}$$

The copies/µL of each sample dilution is then multiplied by the corresponding dilution factor to determine the neat copies/µL. The neat copies/µL of the sample dilutions in which the $C_T$ was within the $C_T$ range of the standard curve are then averaged to determine the copies/µL of the sample.

The % (g/L DNA)/(g/L RNA) (%w/w) of the sample is then calculated using the following equations.

$$(g/L\ DNA) = \frac{(\frac{copies}{\mu L})(Plasmid\ MW\ (kDa))(1 \times 10^6(\frac{\mu L}{L}))}{(6.022 \times 10^{23}\ molecules/mole)}$$

$$\%\,{}^{w}/_{w} = \frac{\text{g/L DNA}}{\text{g/L}\left(\frac{\text{mg}}{\text{mL}}\right)\text{RNA}}$$

**System Suitability and Test Article Acceptance Criteria**

System suitability and acceptance criteria are summarized in Table 31.

**Table 31: System Suitability and Test Article Acceptance Criteria**

| Category | Parameter | Acceptance Criteria |
|---|---|---|
| System suitability | Linearity | CCI [redacted] |
| System suitability | Quantitative Range | |
| System suitability | No Template Control | |
| System suitability | Positive Control | |
| Sample Suitability | Precision | |
| Sample Acceptance | Result Reporting | |

## 3.2.S.4.2.8 pH

CHVI-82391 (Lonza AG) are the methods used to determine the pH of CX-024414 mRNA in accordance with current USP <791>. pH is a numerical scale used to specify the acidity or basicity of an aqueous solution. It is defined as the decimal logarithm of the reciprocal of the hydrogen ion activity, aH+ , in a solution.

**Procedure**

A suitable pH meter, such as the CCI [redacted] Meter or the CCI [redacted] pH Meter (or equivalent), is utilized. These pH meters, including their associated electrodes, are operated in accordance with manufacturer recommendations and meet the instrument requirements listed in USP including pH measurement resolution and ability to compensate for temperature. The pH meter is calibrated (standardized) using commercially prepared, NIST traceable, standardization solutions each day of use using either a 2- or 3-point calibration, as appropriate to bracket the expected pH of samples to be tested. The standardization buffers will span no more than 4 pH units. The calibration must meet slope and offset acceptance criteria. The calibration is also verified using commercially prepared, NIST traceable, standardization solutions before measuring samples. The temperature adjusted pH value of the verification buffer reading must be CCI [redacted] when compared to the label claim. The calibration, verification, and sample measurements are performed at room temperature.

### 3.2.S.4.2.9 Bacterial Endotoxin

CHVI-6303 is used for detection and quantitation of bacterial endotoxin for CX-024414 using a Kinetic Chrmogenic method utilizing an Endotoxin Plate Reader. Testing is performed as outlined in the United States Pharmacopeia, (USP) <85>, Bacterial Endotoxin Test. These chapters are harmonized with the chapters of the same name in the European Pharmacopoeia (EP 2.6.14) and the Japanese Pharmacopeia (JP 4.01). The endotoxin level, measured as endotoxin units (EU) in the DS sample is divided by the CX-024414 mRNA concentration and reported as EU/mg.

### 3.2.S.4.2.10 Bioburden

CHVI-8889 is used to assess bioburden for CX-024414 final Bulk Drug Substance. Testing performed included total aerobic microbial count (TAMC) and total yeast and mold count (TYMC) as outlined in the United States Pharmacopeia, (USP) <61>, Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests. These chapters are harmonized with the chapters of the same name in the European Pharmacopoeia (EP 2.6.12) and the Japanese Pharmacopeia (JP 4.05). All media used in bioburden testing must be qualified prior to use.

CX-024414 samples are prepared using a total volume of CCI for TAMC testing and CCI for TYMC testing of the CX-024414 bioburden using membrane filtration. CCI plates for TAMC are incubated at CCI, and CCI plates for TYMC are incubated at CCI. Bioburden results are reported as CFU per total volume tested for CCI (TAMC) and CCI (TYMC). If no recovery is detected, bioburden results are report as CCI per volume tested.