Abstract

To ensure final product safety, clearance of host cell DNA from the drug substance is essential in the manufacturing process for therapeutic proteins and monoclonal antibody (MAB) drugs. We have developed and validated an automated process for monitoring clearance of Chinese Hamster Ovary (CHO) DNA impurities during the capture and polishing steps of downstream processing. This method, which eliminates manual steps in DNA extraction and subsequent qPCR analyses, can help overcome the analytics bottleneck during process development and routine testing. DNA extraction with Roche’s MagNA Pure LC System offers excellent DNA recovery rates and robustness towards matrix effects for subsequent highly sensitive quantitative PCR. The MagNA Pure LC System eliminates manual dilution of high protein or DNA loads, manual neutralization of samples from acidic protein purification, and manual addition of carrier RNA. Here, we show validation data from in-process control QC samples collected during the pharmaceutical manufacturing process that were spiked with defined amounts of CHO DNA, and from in-process controls, using Roche’s MagNA Pure LC System and Qiagen’s QIAcube Instrument.

For general laboratory use.
Roche’s Penzberg production site is a center of competence for therapeutic proteins (see Figure 1). The focus of therapeutic protein production is the manufacturing of life-sustaining drugs for oncology, anemia and virology.

The removal of host cell protein and host cell DNA during downstream processing of therapeutic proteins is required to guarantee high quality and purity of the biopharmaceutical product (Figure 2). Both process developers and analysts working in quality control have to demonstrate the clearance of DNA impurities from the CHO (Chinese Hamster Ovary) production cell lines.

<table>
<thead>
<tr>
<th>Description</th>
<th>MagNA Pure LC Instrument</th>
<th>QIAcube Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase K digestion included/automated</td>
<td>☹</td>
<td>☹</td>
</tr>
<tr>
<td>No dilution of samples needed</td>
<td>☹</td>
<td>☹</td>
</tr>
<tr>
<td>No carrier RNA needed</td>
<td>☹</td>
<td>☹</td>
</tr>
<tr>
<td>No neutralization of pH needed (~10% acidic samples)</td>
<td>☹</td>
<td>☹</td>
</tr>
<tr>
<td>All reagents contained in kit and ready to use</td>
<td>☹</td>
<td>☹</td>
</tr>
<tr>
<td>Automated PCR Setup</td>
<td>☹</td>
<td>☹</td>
</tr>
<tr>
<td>Run Duration</td>
<td>180 min 32 Samples</td>
<td>90 min 12 Samples</td>
</tr>
<tr>
<td>Less hands-on-time and reagents</td>
<td>☹</td>
<td>☹</td>
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In 1996, the WHO defined a standard value of 10 ng CHO DNA per therapeutic dose.[1] PCR-based DNA quantification of in-process QC control samples is performed to demonstrate the effectiveness of certain purification steps in reducing CHO DNA content. This in-process testing requires reproducible and sensitive methods for DNA extraction with high recovery rates.

DNA extraction of in-process controls is not trivial. Samples can have high protein loads ranging from 8 up to 150 mg/ml and having a range of 0 to 100 ng/ml DNA.

Proteins present can negatively influence recovery of DNA extraction and sensitivity of subsequent PCR analyses.

Samples which are acidic after purification steps using chromatography can also interfere with DNA extraction. In addition, DNA adsorption to surfaces of disposables (e.g., pipette tips) can impair recovery rates and accuracy. To prevent this, some DNA extraction kits require addition of carrier RNA to ensure high recovery rates and high sensitivity in subsequent PCR analyses.

Figure 1: Fermentation at the Roche plant in Penzberg, Germany.

Figure 2: The workflow schematic shows fermentation manufacturing and the chromatographic purification process of a therapeutic antibody. Repeated testing is necessary to demonstrate removal of CHO DNA during the production, which indicates a high quality and high purity of the final drug product. Using Roche systems for automated nucleic acid purification and qPCR, we saw striking advantages compared to using QIAcube DNA purification.
## 2 Introduction

In the present study, we provide data for a new integrated workflow using automated CHO DNA extraction from pharmaceutical samples and PCR sample preparation using Roche’s MagNA Pure LC System, followed by sensitive quantitative real-time PCR analyses. The MagNA Pure LC Instrument is fully automated and could be programmed to prepare PCR mixtures. PCR mixes are transferred to the glass capillaries of the LightCycler® 2.0 Instrument or the multiwell plates of the LightCycler® 480 Instrument (both Roche).

## 3 Materials and Methods

### Samples

Samples containing 1 to 150 mg/ml drug substance from different downstream processing steps of a manufacturing process, and different purification stages for therapeutic proteins using the Chinese Hamster Ovary (CHO) production cell line, were obtained from Roche Production and Development at the Penzberg facility in Germany.

### Nucleic acid extraction

Nucleic acid extraction from in-process QC controls spiked with a final concentration of 0.4 to 40,000 pg/ml CHO DNA, at different protein concentrations (0-150 mg/ml protein), was carried out using the MagNA Pure LC Total Nucleic Acid Kit – High Performance (Roche Diagnostics GmbH, Mannheim, Germany). The MagNA Pure LC 2.0 Instrument was used with the “High Sensitivity” purification protocol, with a sample volume of 100 µl and an elution volume of 100 µl. Each run of 180 min included: 30 samples, a positive control (~ 90 pg/ml CHO DNA in TE buffer), and a negative control (TE buffer only).

To identify optimal conditions, samples were purified with a Proteinase K digestion step, and with and without addition of carrier RNA. For samples obtained from an acidic column based purification step (pH 3.5), DNA isolation was carried out with and without a prior neutralization step. All extractions were performed in replicates.

### Reference method

CHO DNA extraction using the column-based QIAamp Viral RNA Mini QIAcube Kit on the QIAcube Instrument (Qiagen, Hilden, Germany) was used as the reference method. Each run of 90 min included: 10 samples, a positive control (~ 90 mg/ml CHO DNA in TE buffer), and a negative control (TE buffer only). Sample and elution volumes were 140 µl each. Carrier RNA was manually added to the samples (included in the Qiagen kit). For the Qiagen kit, a manual Proteinase K digestion step must also be performed prior to extraction. This Proteinase K digestion was done at +72°C using Proteinase K and SDS. The purification on the MagNA Pure LC instrument includes an integrated Proteinase K step.

Purification was then carried out with and without neutralization prior to the extraction of CHO DNA from samples obtained after the acidic purification step (pH 3.5).

### Quantification of CHO DNA by PCR with LightCycler® System

Quantification of nucleic acids was performed using the capillary-based LightCycler® 2.0 Instrument or the microtiter plate-based LightCycler® 480 Instrument using an external standard curve (4 pg/ml – 400,000 pg/ml of CHO DNA) for absolute quantification (Roche Diagnostics, Mannheim, Germany). Each PCR run included a positive control from the DNA extraction step, and two negative controls. One negative control was for the extraction, and one negative control was for the PCR reaction. A calibration standard for the alignment of the external standard curve (400 pg/ml CHO DNA) was also used. Each amplification reaction of 45 cycles, set up with 15 µl PCR master mix and 5 µl eluate, was run in duplicate.
Determination of linearity

In this exemplary experiment, the linearity for the relevant targeted dynamic measurement in the range of 4 to 40,000 pg/ml of spiked DNA in solutions of therapeutic antibodies was investigated (see Figure 3). Linearity was determined for the different protein concentrations, using an undiluted protein concentration of about 25 mg/ml, as well as dilutions of samples with other types of therapeutic antibodies, thus representing a wide range of protein concentrations (data not shown).

Table 1: Determination of Quantitation Limit (QL) and Detection Limit (DL) (DL) for the MagNA Pure LC Instrument. Results met the target acceptance criteria for standard and high protein concentrations. Real-time PCR was performed using a LightCycler® Instrument.

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Sample [pg/ml]</th>
<th>Sample + DNA Spike DL [pg/ml]</th>
<th>Sample + DNA Spike QL [pg/ml]</th>
<th>Accuracy QL [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>~25 mg/ml Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.06</td>
<td>0.46</td>
<td>3.97</td>
<td>106</td>
</tr>
<tr>
<td>STD</td>
<td>0.11</td>
<td>0.07</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>~150 mg/ml Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.01</td>
<td>0.39</td>
<td>3.97</td>
<td>109</td>
</tr>
<tr>
<td>STD</td>
<td>0.03</td>
<td>0.14</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 3: Samples of a therapeutic antibody solution (25 mg/ml) were spiked to final concentrations of 4, 40, 400, 4,000 and 40,000 pg/ml CHO DNA respectively. The samples were purified using a MagNA Pure LC 2.0 Instrument to examine the linearity of the resulting purified DNA yields.
Determination of accuracy

Nucleic acid extraction using the MagNA Pure LC System demonstrated excellent recovery rates (see Figure 4a). For protein concentrations of 25 mg/ml, spiked with 40, 400 and 4,000 pg/ml DNA, mean accuracy was within the target range of 80-120%. This protein concentration is the representative final concentration in common protein drug formulations.

Using the reference method with the QIAcube, recovery rates were only acceptable when samples were diluted, indicating negative effects from the sample matrix (see Figure 4b).

These findings indicate that quantitative real-time PCR can be negatively affected by protein in the eluate. Manual dilution and Proteinase K addition prior to DNA extraction appears to be required when using the QIAcube Instrument to prevent protein leakage into the eluate, which then negatively affects accuracy and sensitivity of qPCR analyses (see Figure 4c). Note again, that the MagNA Pure LC Total Nucleic Acid Kit – High Performance procedure incorporates an automated step for Proteinase K digestion.

**Figure 4a:** Therapeutic antibody samples (25 mg/ml) were spiked to final concentrations of 40, 400 and 4,000 pg/ml CHO DNA respectively. DNA isolation was performed using the MagNA Pure LC 2.0 Instrument. Results in turquois show acceptance criteria (80-120%) where achieved.

**Figure 4b:** Therapeutic antibody samples (25 mg/ml) were spiked with 423 pg/ml CHO DNA each. DNA isolation was performed using the QIAcube Instrument with the additional manual steps, such as prior Proteinase K digestion. Results in dark gray show acceptance criteria (80-120%) where met, and results in light gray where they were not.

**Figure 4c:** Therapeutic antibody samples with protein content (14-17 mg/ml) were digested with Proteinase K. Undiluted and 1:10 diluted samples were purified using the MagNA Pure LC Instrument and the QIAcube Instrument.
High Protein Loads
For protein loads of 150 mg/ml, DNA extraction using the MagNA Pure LC Instrument showed a mean recovery of 109%. DNA extraction using the QIAcube Instrument required prior dilution and Proteinase K digestion for these samples. However, the results still did not comply with our acceptance criteria of 80-120% (see Figure 5).

DNA extraction using the MagNA Pure LC Instrument did not require further dilution of proteinaceous samples. It produced higher sensitivity and reproducibility in the downstream PCR analyses, especially at the low DNA and high protein concentrations in samples from late protein polishing steps.

Effect of acidic samples
Some of our in-process intermediate products are acidic. Tests examining the need for neutralization to pH 7 prior to DNA isolation were performed using the MagNA Pure LC Instrument. Both samples which were neutralized prior to processing, and samples which were not neutralized showed similar results (see Figure 6a).

When comparing the Roche and Qiagen instruments, samples which were not neutralized prior to processing showed better performance using the MagNA Pure LC Instrument than with the QIAcube Instrument. When performing the neutralization step, the QIAcube Instrument was in a comparable range (see Figure 6b). Based on these and other data (not shown), we found that this additional neutralization step can be omitted when using the MagNA Pure LC Instrument.

Figure 5: In-process control samples of another therapeutic antibody with very high protein content (150 mg/ml) were used undiluted or diluted, and spiked with CHO DNA. These samples were then purified using either the Roche or Qiagen automated sample preparation instruments. Relying on existing data, the MagNA Pure LC Instrument was not tested for 400 pg/ml spike, as it had worked fine earlier. For Qiagen, the low DNA amounts spiked were also not tested, since these did not previously meet the target criteria.

Figure 6a: Acidic in-process control samples of a column purified therapeutic antibody are tested for matrix effects using the MagNA Pure LC Instrument. No manual neutralization step is necessary.

Figure 6b: An acidic in-process-control sample of a column purified therapeutic antibody is tested for matrix effects using the MagNA Pure LC Instrument and compared to the Qiagen Instrument. For the QIAcube a neutralization step is necessary.
4 Results and Discussion

Eliminating unnecessary steps: Effect of adding carrier RNA
Using the MagNA Pure LC Instrument, samples with low DNA concentration (1:50 diluted) for which carrier RNA had been added prior to processing (as recommended by Qiagen for the QIAcube), and samples without carrier RNA showed similar results (see Figure 7). Qiagen recommends adding carrier RNA for a good purification performance of DNA at low concentrations in their instructions for use. This recommendation was confirmed by our experience (data not shown). We were able to omit the step of manually adding carrier RNA when using the MagNA Pure LC Instrument.

5 Conclusion

Nucleic acid extraction using the MagNA Pure LC 2.0 System and MagNA Pure LC Total Nucleic Acid Kit – High Performance provides a robust automated workflow for sensitive and reproducible quantification of host-cell DNA in both in-process controls and final drug substance samples of protein-based drugs. In contrast to the current QIAcube, the MagNA Pure LC 2.0 System required no manual pre-processing, additional Proteinase K, neutralisation or dilution steps, nor did it need the addition of carrier RNA. It additionally offers less hands-on time at the same or higher performance level as the QIAcube instrument. The system provides a fully automated workflow from DNA extraction to DNA quantification on the LightCycler® 2.0 and LightCycler® 480 Instruments. Hence it can help to reduce time-to-analysis, manual work and expedites testing for development, in process control testing and quality control testing. The LightCycler® Instruments provide the attractive option of loading standard curve data, and normalizing results using just an additional single standard control in each PCR run. This option means that it is not necessary to run the many replicate samples required for a standard curve for each new purification and real-time PCR run.

6 References
