New Analytical Methods for Host Cell Residual DNA testing

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New Cells for New Vaccines II
Wilmington, DE, September 17-19, 2007
Content

- Generalities on DNA quantification methods
- Hybridization method: principle, advantages/disadvantages
- Threshold® method: principle, advantages/disadvantages
- Quantitative PCR (qPCR) method: principle, advantages/disadvantages
- DNA Size evaluation by qPCR (Vero DNA example)
- Results/Discussion
- Regulations overview
- Conclusion
Sensitivity/specificity

- UV Spectrophotometry ($A_{260}$): 1 µg/ml, Nucleic Acids
- Colorimetry: 5 µg/ml, total DNA
- Picogreen: 10 ng/ml, double-stranded DNA
- Threshold®: 10 pg/ml, total DNA
- Hybridization: 10-100 pg/ml, Specific (Vero DNA)
- qPCR: 10 pg/ml, Specific (Vero DNA)
Hybridization method
Hybridization: principle (1/2)

DNA probe generated from the host cell DNA

DNA (test article) denatured (to single strands) and immobilized on a membrane (Nitrocellulose or nylon): slot blot or dot blot

Sample probed using host cell labeled DNA (random labeling procedure with a radioactive or fluorescent label)
Hybridization: principle (2/2)

Denaturated labeled probe: contact with the membrane immobilized DNA, probe will bind to the complementary sequence of the host cell DNA

- Signal is proportional to the amount of DNA immobilized on the membrane
- Signal intensity can be compared to spots that correspond to a standard curve (Visual quantification) or signal is measured using a densitometer

Standard curve:
- 1 ng to 0.1 pg/well (Vero DNA)

Sensitivity:
- 10 to 100 pg/ml depending on labeling systems

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Hybridization: Equipements

- Filtration equipment (dot or slot)
- Hybridization oven
- Appropriate laboratory (for radioactive handling and autoradiography)
- Beta counting detector (for radioactive probe labeling step) or densitometer (Phosphor- or fluorescence imaging system) for reading step
Hybridization: Advantages/disadvantages

Advantages:
- Specificity
- Sensitivity (for radioactive labeling)

Disadvantages:
- Low throughput (6 samples per series)
- Pre-treatment or extraction step
- Duration of the test: 7 days (radioactive detection)
- Handling of radioactivity
- Visual quantification if no densitometer (semi-quantitative method)
- Low reproducibility (WHO collaborative study 1992): high degree of variability between laboratories
- Not standardized
Threshold® method
**Threshold® method**

- Immuno-enzymatic method: sandwich ELISA
- pH variation measurement
Threshold® method performances (1/2)

Response intensity and DNA size
- Independent regarding DNA types (Calibration using Calf Thymus DNA)
- Proportionality Response/Mass (Thus to DNA size)
- Proportionality loss for size below 1000 nt
- No response below 80 nt but signal inhibition through reagents consumption
- No more inhibition below 30-40 nt (no detection, no reagents consumption)
All DNA fragments larger than 100 bases (after denaturation) are quantitated.
Threshold® method performances (2/2)

Quantification window
- Narrow (5 to 150 pg / well)
- Very good sensitivity for an ELISA
- Hook effect (Sandwich in one time)
- Systematic spiking (50 pg / well) to overcome the hook effect

Interferencies (detected with the spiking)
- Physical: pH, osmolality
- Chemical: detergents, proteins, solvants, …
Threshold® method: Advantages

- Very sensitive method although not radioactive
- Detect all DNA species
- Accuracy greater than hybridization
- Allow to determine DNA clearance / purification step (Purification design)
Threshold® method: disadvantages

- Sterile sample (without bacterial DNA)
- Inhibition by fragments below 80 nt
- Operating conditions difficult to optimize and sample-dependent
- Narrow quantification range (5 to 150 pg/well)
- Low throughput (15 dilutions / 12 sticks series)
- Only one supplier
- Not applicable to DNA viruses
- No information on the DNA size
Quantitative PCR : qPCR

- Principle
- Application to residual DNA quantification
- Application to DNA size evaluation
Quantitative PCR (qPCR) : principle

- Specific DNA quantification by polymerase chain reaction (PCR) and real-time detection of amplified product by emission fluorescence monitoring
- Detection of a genomic target sequence (Beta-actin for Vero DNA)
- Small fragment (59 bp for Vero DNA)
- Steps: Extraction, qPCR, interpretation
- Detection using a probe (Taqman…)
- Standard curve: 100 ng to 1 pg/reaction
- Limit of detection (qPCR Vero): 10 pg/ml
- Possibility to use an internal control to evaluate the reaction yield (Duplex qPCR)
The internal control (IC) is a synthetic DNA:

- Added to each sample, before extraction step, at a known quantity
- Amplified simultaneously with the genomic target (using the same primers set)
- Separate DNA quantifications of the target DNA and of the IC (2 different probes for detection with to different reporters)
- In compliance with European Pharmacopeia chapter (2.6.21)

The IC allows **extraction + PCR yield** evaluation for each sample.
**Internal control 2/2**

MGB probe (FAM)

Reverse primer → Forward primer

Genomic Vero DNA

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MGB probe (VIC)

Reverse primer → Forward primer

Plasmid sequence Vero Sequence IC sequence Vero Sequence Plasmid sequence

Internal control 301 bp
Advantages:

- Specific detection (Viral DNA/cellular DNA), sensitive (theoretical: 10 pg/ml) and fast (2 days for extraction, amplification and analysis)
- Single-stranded and double-stranded DNA detected up to the defined size
- High throughput (Up to three extractions can be run simultaneously = up to 70 samples/qPCR)
- Possibility to run an internal control to evaluate the extraction + PCR yield of the sample by duplex qPCR (correction of the result by calculation possible)
- Broad range of detection: 100 ng/reaction to 1 pg/reaction

Disadvantages:

- Do not quantify fragments below the size of the PCR product (59 bp for Vero DNA)
Vero DNA size Evaluation using qPCR
Viral vaccines under development produced on Vero cells

Internal discussions and comments from the VRBPAC suggest the need for continued concern about the level of residual Vero cell DNA in products manufactured in these cells. Although the World Health Organization (WHO) currently accepts a limit of residual DNA from continuous cell lines of 10 ng per dose for these products when administered parenterally, CBER wishes to continue considering the level of risk posed by residual Vero cell DNA on a case-by-case basis for viral vaccines. Consideration will also be given to the method of vaccine administration, e.g., parenteral, mucosal, or other route. Based on this concern, CBER recommends that you:

a. Measure the amount and size distribution of residual cellular DNA in your final product if you have not done so already. Please submit these results to your IND or MF and describe them in terms of the amount of residual cellular DNA per human dose of final formulated vaccine.

b. Consider various methods (e.g., DNase treatment) by which the amount and size of residual cellular DNA might be further reduced. Please comment on what you have done or intend to do to consider the introduction of additional DNA reducing methods into your process, as well as the potential impact of such changes on the performance (e.g., immunogenicity) of the product.
Five primer sets which amplify overlapping fragments of Vero DNA: 59 bp, 108 bp, 240 bp, 407 bp and 620 bp

Evaluation of the relative proportion of each DNA fragment population in samples

Designed on Beta-actin gene
Principle

5’- 620 bp -3’

176 FP620 389 FP407 556 FP240 737 FPMGB 756 MGBOV 795 RPMGB 844 RP108

59 bp

240 bp

407 bp

620 bp

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Final operating conditions

- Synthetic Vero DNA reference (800 bp)
- Five logs standard curve: $10^5$ to 10 copies / 10 µl
- 45 cycles
- Internal control for evaluation of extraction + PCR yield (correction)
- PCR efficiency measurement for each size system (correction)
- Calculation of relative percentage for each size/ 59 bp size (100%)
### SARS vaccine: results

<table>
<thead>
<tr>
<th>Vaccine/Step</th>
<th>Batch</th>
<th>Threshold</th>
<th>qPCR VERO</th>
<th>Size evaluation (qPCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pg/ml</td>
<td>pg/ml</td>
<td>CV%</td>
</tr>
<tr>
<td>Purified bulk</td>
<td>1</td>
<td>≤770</td>
<td>380</td>
<td>18%</td>
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<td></td>
<td>2</td>
<td>≤770</td>
<td>270</td>
<td>28%</td>
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<td></td>
<td>3</td>
<td>≤410</td>
<td>260</td>
<td>7%</td>
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<td></td>
<td>4</td>
<td>≤410</td>
<td>200</td>
<td>36%</td>
</tr>
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</table>

**ND:** not detected

**IG:** below the last point from the standard curve
### Rabies vaccine results

#### Step Batch

<table>
<thead>
<tr>
<th>Step</th>
<th>Batch</th>
<th>Threshold pg/dose</th>
<th>CV%</th>
<th>qPCR VERO pg/dose</th>
<th>CV %</th>
<th>Size Evaluation by qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>59 bp</td>
</tr>
<tr>
<td>Filled Product</td>
<td>1</td>
<td>7 000</td>
<td>12%</td>
<td>8 400</td>
<td>19%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7 500</td>
<td>0%</td>
<td>9 500</td>
<td>5%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9 000</td>
<td>6%</td>
<td>17 750</td>
<td>15%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9 500</td>
<td>14%</td>
<td>20 000</td>
<td>3%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7 000</td>
<td>17%</td>
<td>13 500</td>
<td>6%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8 000</td>
<td>9%</td>
<td>19 000</td>
<td>1%</td>
<td>100%</td>
</tr>
<tr>
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<td>7</td>
<td>8 000</td>
<td>30%</td>
<td>20 500</td>
<td>2%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8 500</td>
<td>12%</td>
<td>19 000</td>
<td>24%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>9 500</td>
<td>24%</td>
<td>19 000</td>
<td>10%</td>
<td>100%</td>
</tr>
</tbody>
</table>

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**IG**: below the last point from the standard curve
## Benchmarking study on Rabies vaccines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hybridization</th>
<th>Threshold</th>
<th>qPCR</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/dose</td>
<td>pg/dose</td>
<td>pg/dose</td>
<td>59 bp 108 bp 240 bp 407 bp</td>
</tr>
<tr>
<td>A</td>
<td>[300 - 2 500]</td>
<td>≤ 58 400</td>
<td>185 000</td>
<td>100% 5% IG ND</td>
</tr>
<tr>
<td>B</td>
<td>[300 - 3 000]</td>
<td>130 000</td>
<td>610 000</td>
<td>100% 10% IG ND</td>
</tr>
<tr>
<td>C</td>
<td>[300 - 750]</td>
<td>246 000</td>
<td>43 300</td>
<td>100% 27% IG IG</td>
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<tr>
<td>D</td>
<td>[750 - 5 000]</td>
<td>146 000</td>
<td>74 000</td>
<td>100% 85% IG IG</td>
</tr>
<tr>
<td>E</td>
<td>[100 - 1 000]</td>
<td>≤ 5 800</td>
<td>21 300</td>
<td>100% IG IG ND</td>
</tr>
<tr>
<td>F</td>
<td>[300 - 1 000]</td>
<td>≤ 9 000</td>
<td>25 000</td>
<td>100% 11% 4% IG</td>
</tr>
<tr>
<td>G</td>
<td>[1 000 - 2 000]</td>
<td>7 000</td>
<td>13 500</td>
<td>100% 48% 5% 1%</td>
</tr>
</tbody>
</table>

ND : not detected

IG : below the last point from the standard curve
Possibility to evaluate the residual DNA content, in vaccines produced on Vero cells, using qPCR with two types of information:

- Quantitative
- Qualitative (fragments size distribution)

Results Interpretation should be done by taking into account both aspects.

Hybridization method underestimates the DNA content (Ratio 10 to 500 between hybridization versus qPCR or Threshold, Ratio less than 6 between Threshold and qPCR).

Hybridization method has been shown to be variable between laboratories (WHO collaborative study, Biologicals (1992), 20: 73-81).

qPCR Method more appropriate than Threshold® method for vaccines under development treated with DNase (or containing small DNA fragments following purification process and inactivation process).

Specifications should be based on these qualitative aspects and should be set up according to analytical method used to determine the DNA content.
# Current Regulation status / 2007

<table>
<thead>
<tr>
<th>Regulation</th>
<th>WHO</th>
<th>Eur. Ph.</th>
<th>FDA</th>
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<tbody>
<tr>
<td><strong>Cell substrates monograph</strong></td>
<td>10 ng/dose</td>
<td>10 ng/dose</td>
<td>10 ng/dose (Vero DNA) + size distribution</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>“Characterization and qualification of cell substrates and other biological starting materials used in the production of viral vaccines for the prevention and treatment of infectious diseases”</td>
</tr>
<tr>
<td><strong>Rabies Monograph</strong></td>
<td>10 ng/dose (DNA assay is country-dependent)</td>
<td>Chapter 0216 (2007)EP 5.8</td>
<td>10 ng/dose</td>
</tr>
<tr>
<td>Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs Oct 2005</td>
<td></td>
<td>10 ng/dose</td>
<td></td>
</tr>
</tbody>
</table>
Experimental data demonstrated that only small size fragments are found in our Vero Rabies vaccine (sequential action from the cell lysis by rabies viruses, from purification/inactivation processes):

- Majority of small fragments
- No quantifiable DNA strand higher than 620 bp
- Chemical modifications of DNA
- Non tumorigenic Vero cells (demonstrated using immunodepressed newborn rats)
- Significant safety margin for a vaccine containing DNA quantities in the range of 10 ng/dose
In our hands, Hybridization method underestimates DNA content and is not appropriate to Rabies vaccines produced on Vero cells (which all contain small DNA fragments).

For Threshold® method, interferences are observed with vaccines treated with DNase, containing thus mainly small DNA fragments.

Highest DNA content are usually observed with qPCR method, which is appropriate for vaccines treated with DNase. Moreover, qualitative data regarding size are obtained with this method.

Current regulations should be adapted in order to define DNA content specifications adapted/associated to analytical methods used to quantify DNA content.