Baculovirus Reference Material Initiative

Kuopio, Finland, 08-27-2010
Document for discussion
BRMWT
BRM-discussion

- Molecular design
- Bulk Production
- Downstream processing
- Quantitation & Characterization
- Stability issues & Vialing
- Repository & Distribution
- Logistical issues
- Next steps
Baculovirus Construct

• Construction Strategy
  – Long term stability
  – With/out transgene and which transgene?
  – Baculovirus/BacMam
  – Which mammalian promotor ? CMV?

• Other considerations ?
BRM bulk manufacturing

- Target titer?
- Cell line? Medium?
- Scale of production?
- Batch vs Fed/batch?
- Concentration?
- Freezing?
- Storage and Shipment issues?
Downstream Processing

• Methods ?
• Mode of Storage ?
• Vialing ?
  – Titer per vial
  – Formulation
• Stability studies
  – Accelerated study
  – Long term studies
BRM quantitation and characterization

• Selection of primary methods to determine
  – Infectious units
  – Transducing units (?)
  – Total particles
• Other secondary methods
• Further characterization
  – NSEM
  – Aggregation (?) DLS
• Statistical analyses
• to achieve a good statistical significance of the absolute titer to attribute to the BRM.
Communication & Logistical issues

- Engagement of participating labs - mode of action
- Maximization of the number of labs to quantitate and characterize the BRM
- Participation through donation of validated protocols (assays) or consumables
- Documentation and Reporting (Batch Records)
- Communication between participating labs
- Transportation
- Costs of goods and financial issues
- Identification of a repository and distributor
Baculovirus Molecular Design

A Vaccine Company for the 21st Century

“Making products where speed, cost and safety matter”
Baculovirus Infection in Cultured Cells

Early to Late Phase

Very Late Phase

Factors influencing High level protein expression

- The shut off of early/late baculovirus and host gene transcription
- A high concentration of unpackaged viral DNA
- Efficiency of the baculovirus RNA polymerase
- The biosynthetic capacity of insect cells
Generation of Recombinant Virus

**Polyhedrin or p10 promoter**

+ 

**Gene of Interest (GOI)**

1. Transposition / bacmid
2. Recombination / linearized DNA

10kb

**Transfer vector**

130kb

**Recipient virus genome**

1. Bacmid
2. Linearized Δorf1629 DNA
3. Δorf1629 bacmid

**Recombinant virus genome**
**Bacmid system**

AcMNPV cloned into artificial bacterial chromosome

Recombination in *E. coli* by transposition

Virus obtained by transfection of insect cells with bacmid DNA

**Advantages:**
- Speed
- Allows backbone modifications

**Disadvantage:**
- Instability of r-virus
- Bacmid can grow in insect cell

From Dr. van Oers

(Luckow et al. 1993) Bac-to-Bac system
Linearized baculovirus genomes

- Essential gene, ORF1629, removed by restriction digestion
- Non-infectious linear DNA
- Complemented by transfer vector

- Up to 98% recombinant virus
- Little or no rounds of (plaque) purification required

From Dr. van Oers

(Kitts et al. 1993)
BaculoBlue / Baculogold systems
Infectivity in insect cells is restored by homologous recombination with a transfer plasmid containing GOI.
BAC replicon replaced by gene of interest (GOI) improves the virus stability

The science of baculovirus expression™

Dr. King (OET) with modification
**Viral Genome without non-essential genes**

**Genes deleted in commercially available viral genomes**

- **Chitinase**
  - Involved in liquefaction of insects – blocks the progress of r-proteins through ER
- **Viral Cathepsin (v-cath)**
  - Involved in liquefaction of insects – degrades r-proteins
- **p10**
  - Very late protein – competes with r-protein promoter
- **p74**
  - Required for infection *in vivo* – removal improves bio safety of r-virus
- **p26**
  - Function unknown
  - **Egt**
  - Involved in delay of insect molting
### Viral Genome without non-essential genes

#### Other genes non-essential for *in vitro* infection

<table>
<thead>
<tr>
<th>Function of gene product</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODV envelope proteins</td>
<td><em>odv-e26 (Ac16), odv-e66 (Ac46)</em></td>
</tr>
<tr>
<td><em>Per os</em> infectivity factors</td>
<td><em>pif-1 to 3 (Ac22, 115, 119), Ac145, Ac150</em></td>
</tr>
<tr>
<td>Polyhedron envelope</td>
<td><em>Ac131</em></td>
</tr>
<tr>
<td>Polyhedron morphogenesis</td>
<td><em>Ac68</em></td>
</tr>
<tr>
<td>Fusion protein (inactive)</td>
<td><em>Ac23</em></td>
</tr>
</tbody>
</table>
Design of deletion of the gene

- Compact gene organization
- Overlapping transcripts

Crucial to retain expressions of neighboring genes

1. Deletion of the ORF
2. Partial deletion of the ORF
3. TSS inactivation

Gene
Transcript
Design of Baculovirus Transfer Vectors

Signal peptide: Secretion

Polyhedrin or p10 promoter

Gene of Interest

Tag

Cleavage site: Purification

Reporter/chimeric gene

Fusion or Non-fusion

[Flp-In Sf9 cells]

eGFP

mCherry

Protein Sciences CORPORATION
## Baculovirus Transfer Vectors

<table>
<thead>
<tr>
<th>Tag/cleavage site</th>
<th>Fusion/marker</th>
<th>Secretary signal</th>
<th>Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyhedrin locus–based</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single baculovirus promoter vectors (including LIC vectors)</td>
<td>6xHis, GST, S / factor Xa, thrombin</td>
<td>GUS, GFP, lacZ</td>
<td>gp64, Placental AKP, Melittin, viral chit</td>
</tr>
<tr>
<td>Multiple baculovirus promoter vectors (polyhedrin &amp; p10)</td>
<td></td>
<td></td>
<td>GUS</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bacmid expression vectors (polyhedrin &amp; p10)</strong></td>
<td>6xHis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Burst Sequence of Very Late Genes**

Burst sequence: AT-rich seq between TAAG and ATG

- AT-rich seq between TAAG and ATG
- Polyhedrin / p10: AT ≈ 90%
- 104 TAAG in 186 AcMNPV TSS: AT ≈ 80 to 60%

**Polyhedrin / p10**: ATG

**AT content, %**

- At each nt position
- 10-moving average

**Position from TSS**
Effect of Burst Seq Repeat on GUS expression

BmNPV polyhedrin Burst Seq repeat constructs

Manohar, et al, accepted
Ao38, a new cell line from eggs of the black witch moth (Lepidoptera: Noctuidae)

β-galactosidase [intracellular]

- Ao38 / Serum+
- Ao38 / Serum free
- High Five / Serum+
- Sf9 / Serum+

SEAP [secreted]

- Ao38 / Serum+
- Ao38 / Serum free
- High Five / Serum+
- Sf9 / Serum+

Serum free media; Sf900III: Serum contained (+) media; TNMFH
Acknowledgments

Slides:
Dr. Monique van Oers  Wageningen Univ., Netherlands
Dr. Linda King  Oxford Brookes Univ./OET, UK

References:
1. Baculovirus molecular biology: G. Rohrmann
2. Overview of baculovirus expression system: Murphy et al.
3. TSS/Ao38 studies: G. Blissard
Production of Baculovirus

O.-W. Merten
Agenda

• Culture systems
• Feeding strategies, if needed
• MOI, TOI, etc.
• Questions and suggestions
Production systems:

• **Small scale culture systems:**
  – Plates, T-flasks – e.g.:
    - Advantages: for screening, cloning, selection purposes
    - Disadvantages: cells (Sf9) grow adherently – problems with respect to cell detachment and passaging; only small scale use
  – Spinner, shaker bottles:
    - Advantages: suspension culture of insect cells
    - Disadvantages: limited scalability
Production systems:

• Large scale culture systems:
  – Stirred tank reactor (STR)

    **Advantages:**
    - controlled environment
    - almost illimited scalability
    - all process/feeding modes applicable
    - stainless steel and disposable versions available
    - good mass transfer
    - generally used for the insect cell baculovirus system (volume max.: >500 L)

  **Disadvantages:**
  - more complex handling than for Wave reactor

  ![Stirred tank reactor](image)

  – Wave reactor

  **Advantages:**
  - controlled environment possible
  - easy handling when compared to STR
  - used for baculovirus generation and the production of biologicals using the insect cell/baculovirus system (volume max.: <500 L)

  **Disadvantages:**
  - limited scalability (volume max. < 500 L) – limited mass (O₂) transfer
  - no high cell density process possible – limited mass (O₂) transfer

  ![Wave reactor](image)
Feeding strategies:

- **Batch** – classical production mode
- **Fed batch** – generally needed when high cell density processes are performed

Feeding strategies:
- Continuous feeding
- Medium replenishment before infection
- Selected nutrient supplementation at the TOI or at several time points (best choice for high cell density processes)

**P.** Concentration of waste products

**X.** Viable cell concentration

**S.** Concentration of nutrients

(Mena et al. 2010)
MOI, TOI, etc.

• **MOI**
  – Low (e.g.: 0.1) – reduction of the risk of generating defective particles
  – Use of plaque purified viral seed stock

• **TOI**
  – 72 h p.i.

• **Characterisation**
  – Titer (minimal titer: $10^8$ PFU/ml)
  – Defective particles
  – … - to be discussed in detail

• **Etc.**
Questions and suggestions

• Questions:
  – Which baculovirus to be produced: wt or recombinant (e.g. GFP)
  – Which quantity of baculovirus has to be produced with which quality (purified and formulated ↔ non-purified)
    → criteria for the choice of the production system (spinner/shaker ↔ STR/Wave) and eventually of the production mode
  – Who produces
  – Analytics – quality control?

• Suggestions:
  – Low cell density process (infection at $10^6$ c/ml)
  – Batch process
  – Low MOI (0.1)
  – Time of harvest: 72 h p.i.
BACULOVIRUS
REFERENCE MATERIAL
workshop

Purification/storage

Paula ALVES
IBET, Portugal

Advanced Symposium and EMBO Practical Course
August 26th – 29th 2010
Kuopio, Finland
Downstream Processing for Baculovirus Reference Material

- do we need DSP?
- it is necessary to purify? or just a concentration step?

depends on the application
(quality requirements: identity, strength, purity, potency)

**baculo as a reagent**
for production (proteins, VLP’s...) – requires well characterized master and working seed banks

*versus*

**baculo as a product**

**issue: effect of purification in baculovirus stability (during storage)**
“well characterized” should reflect the entire process, not just the end product

lessons from Adv and AAV

*baculo as a reagent* it is critical to *characterize it and store* it properly:

• raw material testing is important at each stage of production
• harmonization of *analyticals* is critical (e.g. importance of MOI for co-infection strategies for VLPs and viruses e.g. AAV production)
Downstream Processing for Baculovirus reference material

• Methods ?

• Mode of Storage ?

• Vialing ?
  – Titer *per* vial
  – Formulation

• Stability studies
  – Accelerated study
  – Long term studies
A “disposable” and scalable process @ IBET

Bioreactor: e.g. wave or stirred tank

Cell/debris removal
Depth filters (disposable): 3 μm + 0.65 μm

Concentration
UF membranes (can be used disposable): 100 kDa cassettes

Capture/Polishing
Membrane adsorbers (disposable): Sartobind D (DEAE)

Process:
- Viral vector bioreaction bulk (A)
- Microfiltration
- Ultrafiltration/Diafiltration (B)
- DEAE AEXc (C)
- Purified viral vector

Titers up to $10^{10}$ pfu/mL

Clarification:
- TP/IP: 87
- Recovery yield of IP$^{b,c}$ (%): 98 ± 2

Ultrafiltration:
- TP/IP: 53
- Recovery yield of IP$^{b,c}$ (%): 70 ± 5

AEX membrane:
- TP/IP: 18
- Recovery yield of IP$^{b,c}$ (%): 65 ± 5

Desalting:
- TP/IP: NA
- Recovery yield of IP$^{b,c}$ (%): 95 ± 3

Sterile filtration:
- TP/IP: 18
- Recovery yield of IP$^{b,c}$ (%): 90 ± 10

Overall:
- TP/IP: 18
- Recovery yield of IP$^{b,c}$ (%): 38 ± 5

Vicente et al (2009), Gene Therapy, 16:766–775
**Final Product Characterization @IBET**

**Baculoviral Vectors**

- gp64 glycoprotein trimers
- viral DNA
- vp39 capsid
- host cell derived bilipid envelope

**Surface properties**

- Lipid membrane
- Envelope glycoprotein projections
- Heterogeneous charge

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP/IP</td>
<td>&lt; 10:1</td>
</tr>
<tr>
<td>Protein removal</td>
<td>&gt; 80 %</td>
</tr>
<tr>
<td>hc DNA removal</td>
<td>&gt; 95 %</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Comparable with traditional process</td>
</tr>
<tr>
<td>Endotoxin levels (LAL)</td>
<td>&lt; 10 EU/ml</td>
</tr>
<tr>
<td>Size by DLS</td>
<td>(90-100nm x 300 – 450 nm)</td>
</tr>
</tbody>
</table>
**Baculovirus Reference Material**

**Implications to DSP**

- Final purity (impurities intrinsic to the system, adventitious virus)
- Final Concentration (10^8 - 10^9 pfu/mL?)
- TP/IP (generally < 30)
- Aggregation (responsible for the loss of infectivity: >10^10 pfu/mL)
- HCP (< 0.5 ng/mL – BSA as reference)
- Residual DNA (<10 ng/dose, fragments up to 200 bp)
- Endotoxins (<.15 EU/mL)

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**Baculoviruses Storage/Formulation issues**

(only very scarce information is available)

? is purification affecting stability (removal of proteases but also of stabilizers)?

- buffer/pH versus Culture medium; include sucrose/trehalose
- temperature (4ºC versus -80ºC)
- effect of Light
- aggregation
Quantification & Characterization of Baculovirus vector

Amine Kamen, PhD
Kuopio, Finland, 08-27-2010
• Indirect methods to evaluate baculovirus infectivity and infer titers
• Addition of several baculovirus dilutions to either Sf9 or Sf21 cell cultures
• Reading of post-infection events such as
  • cytopathic effects
  • viral protein (ELISA-GP64)
  • marker gene expression (GFP, β-galactosidase, other markers)
  • transgene expression
  • PCR of viral genome
  • cell growth cessation
• Baculovirus titer determined
  • plaque assay
  • end-point dilution assay
  • units are plaque forming units/mL
• In the case of BacMam
  – Post-events of transduction of mammalian cells, for example HEK-293 are recorded.
  – Similar procedures to infection cell based assays might be used
Result reading:
(Reed-Muench method)

TCID\textsubscript{50} : end-point dilution which presents 50\% of infected wells.

| Proportional Distance (à 50\%) | \[
\frac{(% >50\%) -50\%}{(% >50\%) - (% <50\%)} \]
| \[
\frac{66.7\% - 50\%}{66.7\% - 16.7\%} \]
| = 0.3

Log ID\textsubscript{50} = (log dil. >50\%) + (Prop. dist. * log (dil. factor) )
= (-6) + (0.3 * log(5) )
= -5.8

ID\textsubscript{50} = 10^{-5.8}

Log dilution:
[-2, -3, -4, -5, -6, -7, -8, -9, -10]

% infected wells:
100 66.7 16.7 0

Example
TCID\textsubscript{50} Assay

Cytopathic effect
(cells present a morphologic change
Due to viral infection)
### Baculovirus Infectivity Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Type of assay</th>
<th>Duration of assay</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytopathic effects</td>
<td>Plaque assay or End-point dilution</td>
<td>4-7 days</td>
<td>Posse and King 1992 O’Really, Miller and Luckow 1994</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>Plaque assay or End-point dilution</td>
<td>4-7 days</td>
<td>Sussman et al., 1995</td>
</tr>
<tr>
<td>GFP</td>
<td>Plaque assay or End-point dilution</td>
<td>4-7 days</td>
<td>Cha et al., 1997</td>
</tr>
<tr>
<td>MTT</td>
<td>End-point dilution</td>
<td>6 days</td>
<td>Mena et al., 2003</td>
</tr>
<tr>
<td>Alamar blue</td>
<td>End-point dilution</td>
<td>24 h</td>
<td>Poulquen et al., 2006</td>
</tr>
<tr>
<td>Cell size</td>
<td>End-point dilution</td>
<td></td>
<td>Janakariman et al., 2006</td>
</tr>
</tbody>
</table>
Fig. 1. Overview of recombinant baculovirus titration methods and time required to perform each method.
Total particles/BV Genomes

- Direct methods to measure the amount of baculovirus based on DNA labeling or vector genomes quantification
- Does not provide information on baculovirus infectivity

<table>
<thead>
<tr>
<th>Method</th>
<th>Total particle quantification</th>
<th>Time (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry</td>
<td>Total particle quantification DNA labeling (VP/mL)</td>
<td>4</td>
<td>Shen et al., 2003</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td>Total particle quantification Specific genome sequence targeted (VG/mL)</td>
<td>3</td>
<td>Vieira et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hitchman et al., 2007</td>
</tr>
</tbody>
</table>
Quantitation of baculovirus particles

Virus stock is diluted in PBS buffer

Virus is fixed with paraformaldehyde

The sample is frozen-thawed to increase virus permeability

Triton X-100 is added to enhance permeability

Virus is stained with SYBR Green I to increase fluorescence

FACS analysis

The entire process takes 2 hours

Viral double stranded DNA labeling by Cyber green. Shen et al. (2002), J. Virological methods 105:321-330
Other methods for characterization

- NSEM
- DLS
- HPLC
- Total proteins
- Western
- UV detection
- Others: