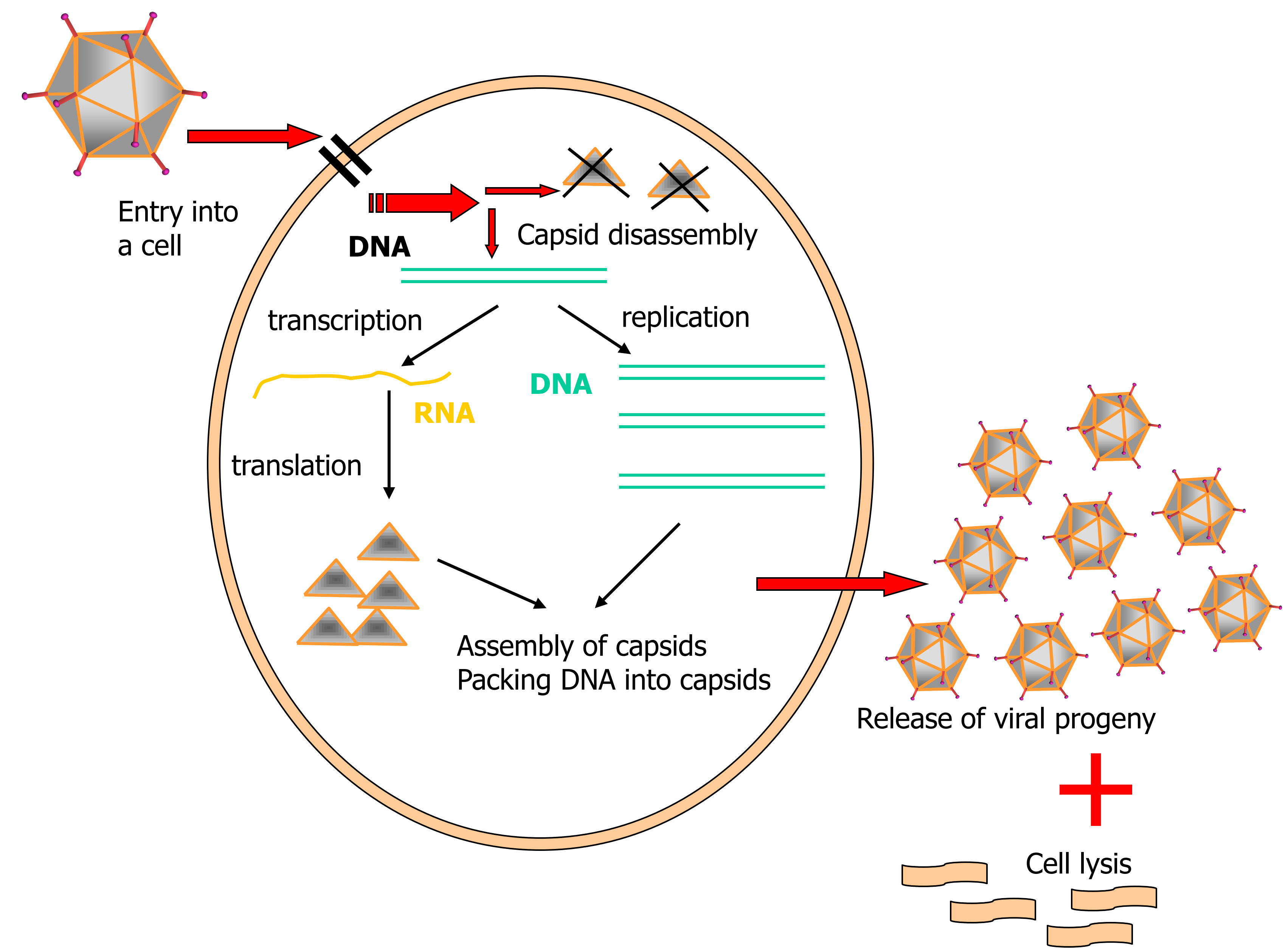
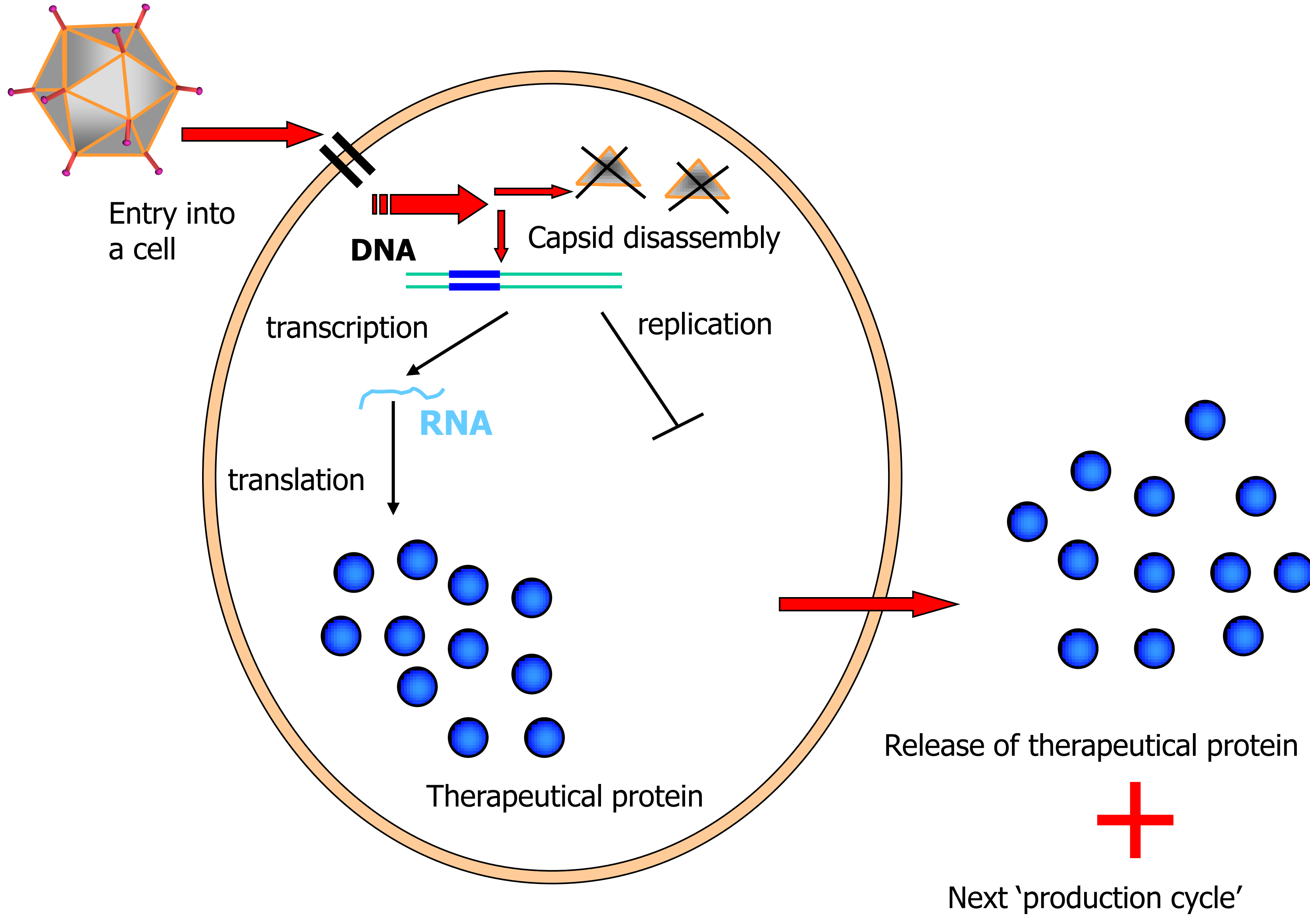
Adenoviral vectors

Adenoviruses

Adenovirus's (Ad) infection



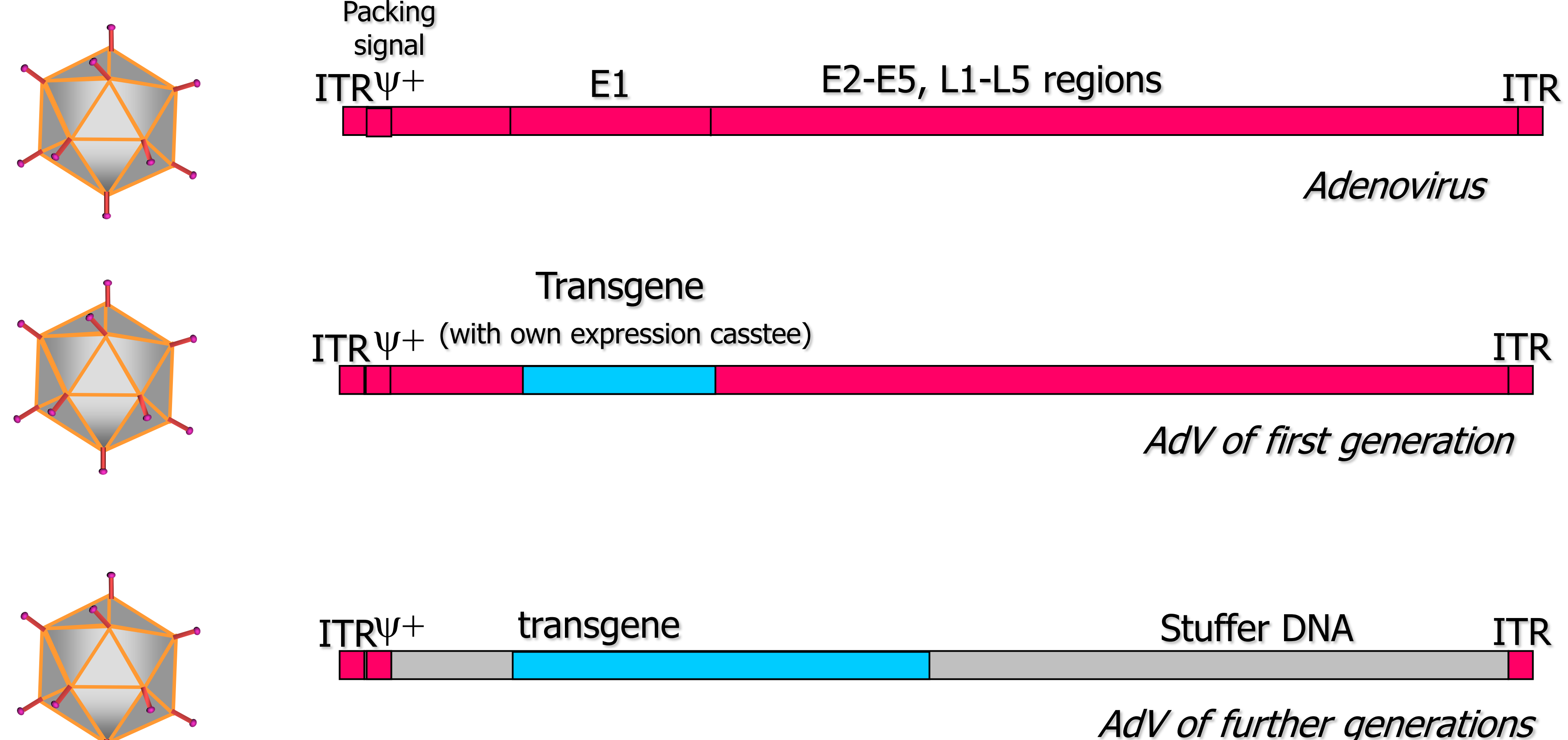
Adenoviral vector's (AdV) infection



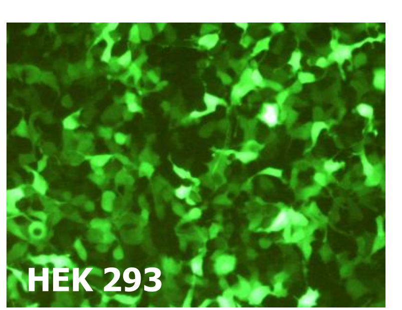
Genome

* **Genome: 36 kb double-stranded linear DNA consisting:**
    - **Early genes (responsible for viral gene transcription and DNA replication)**
    - **Late genes (coding proteins required for virus assembly)**

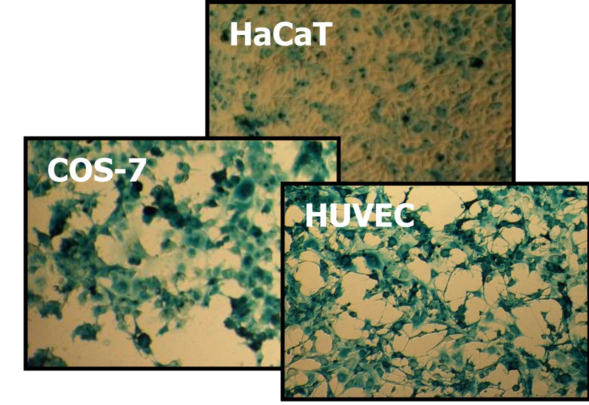
* **E1 early genes (5 kb) are essential for the subsequent adenoviral gene expression**



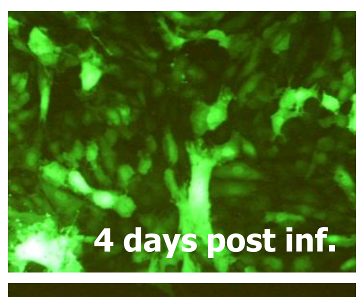
Pros and cons of AdV

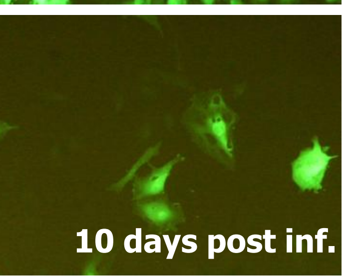


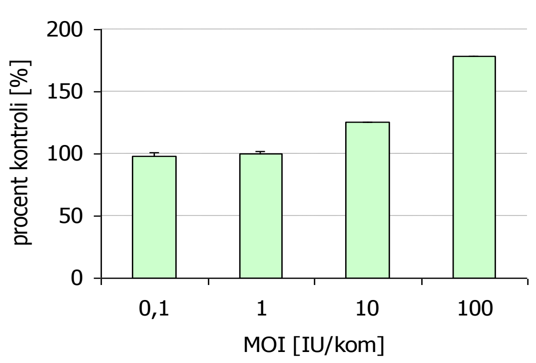
+ Efficient transduction and high transgene expression
+ Infection of many cell types, dividing and no-dividing
+ Easily produced to high titers



Ad-β-gal = $2*10^8$ IU/ml
Ad-GFP = $5*10^9$ IU/ml
Ad-HO-1 = $5*10^9$ IU/ml
Ad-helper = $2*10^{10}$ IU/ml





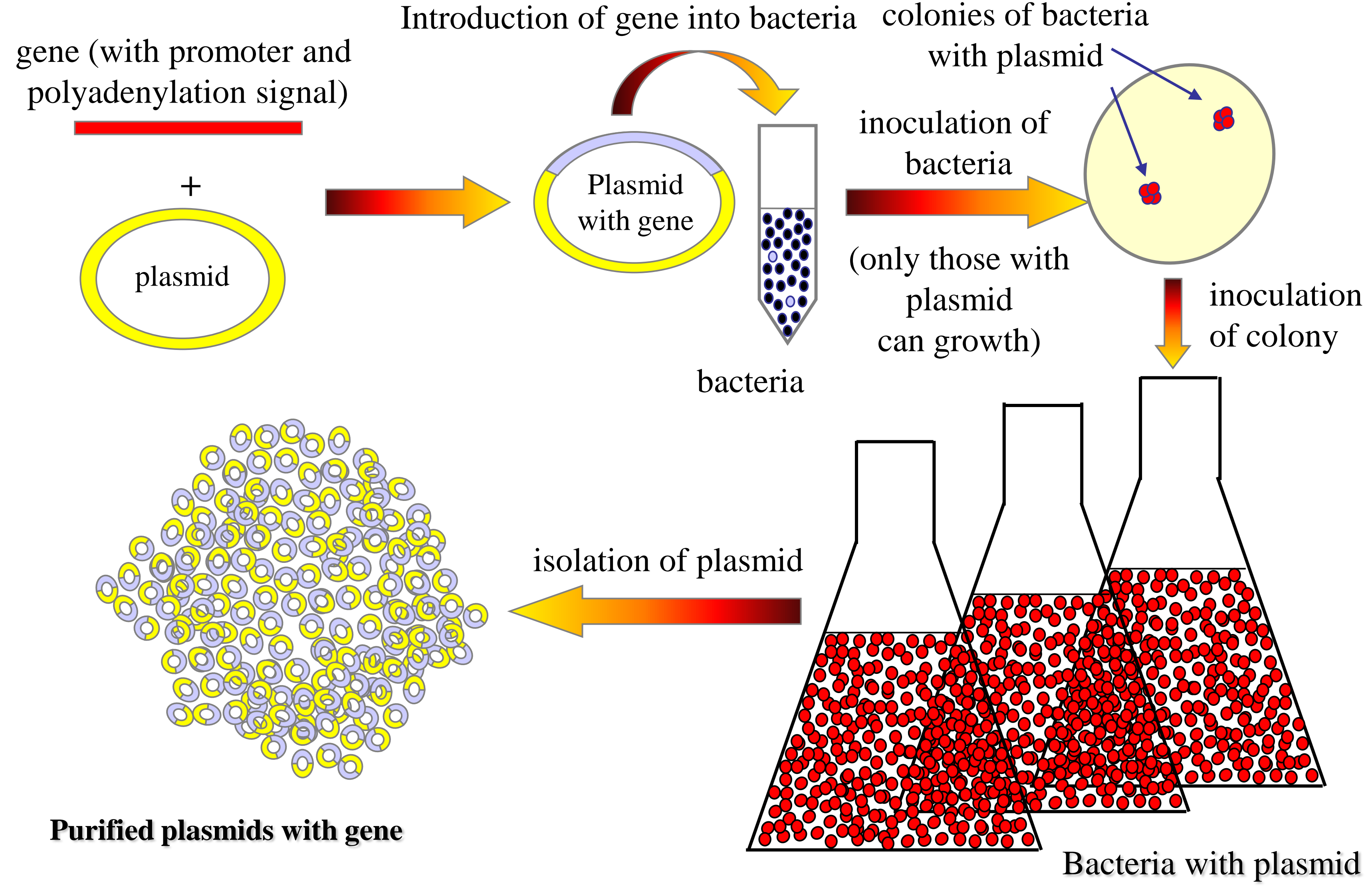


- Cytotoxic and immunogenic
- Short transgene expression

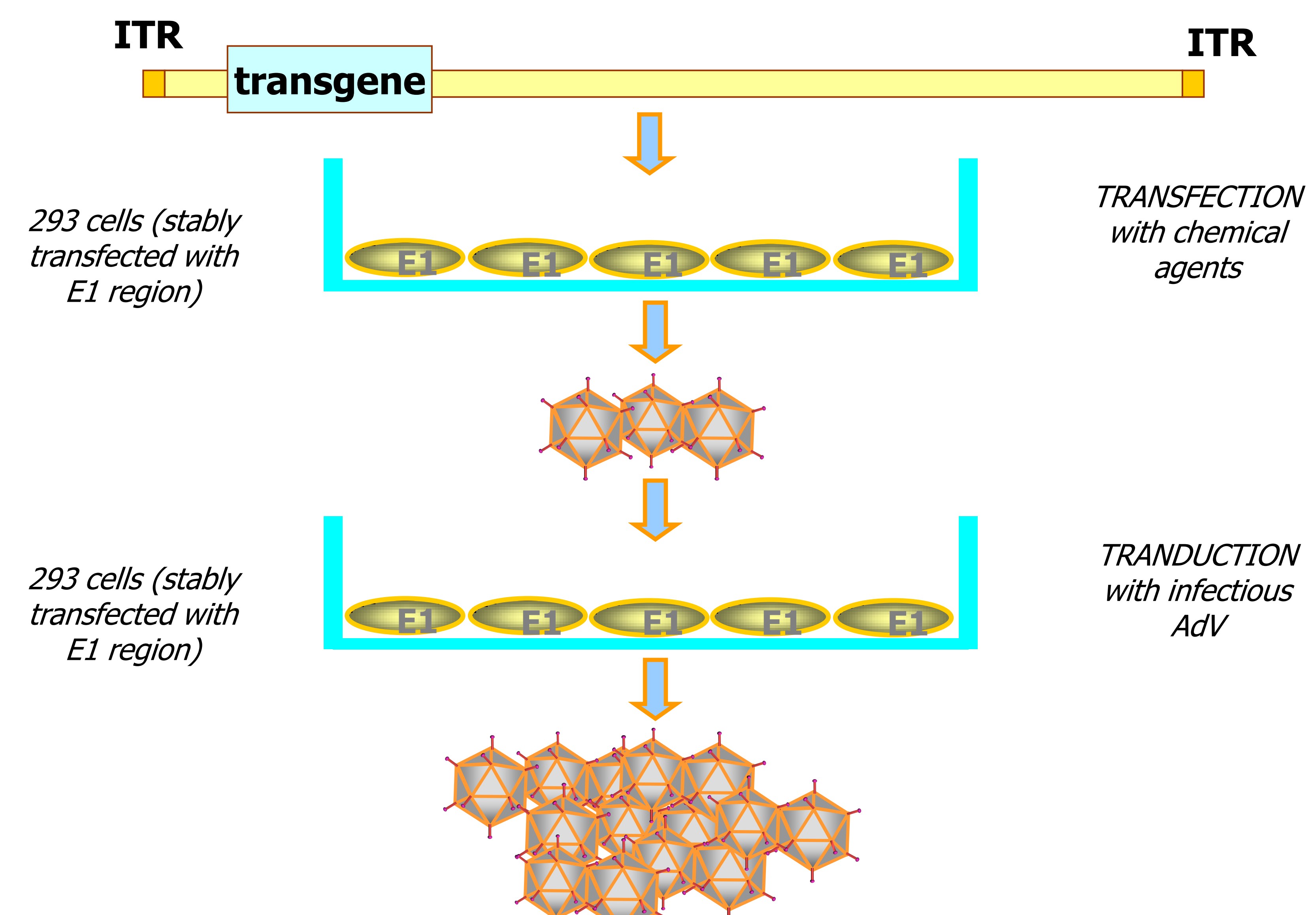
	AAV	RV	AdV
Easy and cheap production		✓	✓
High titers			✓
No cytotoxity and immunogenity	✓	✓	
No risk of oncogenesis	✓		✓
Stable transduction		✓	
High transduction efficacy		✓	✓
Transduction of dividing and no dividing cells	✓		✓
High transgene capacity			✓*

Let's move to practice

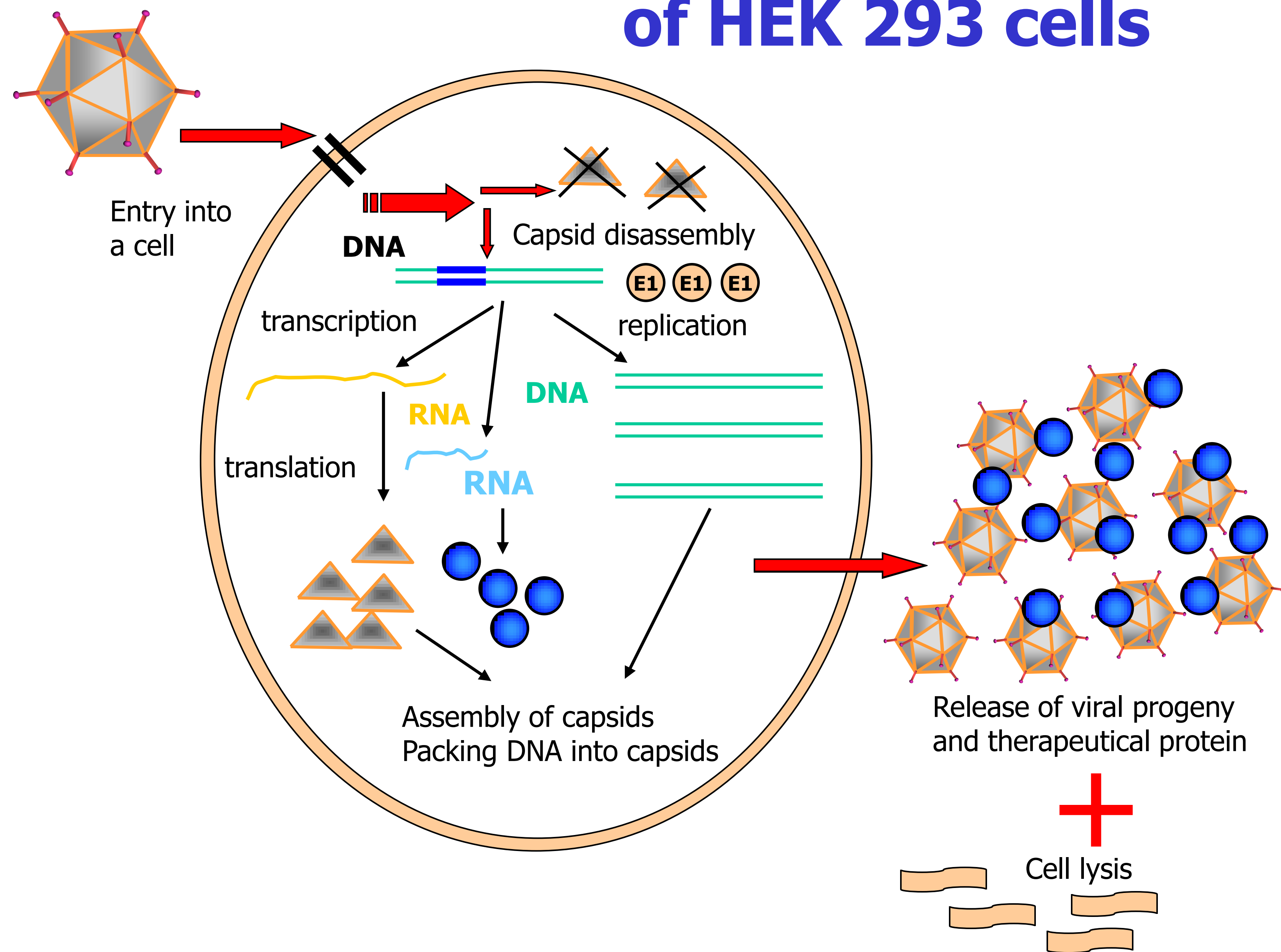
I: Plasmid construction and amplifiation



II: Production of AdV in packeging cells

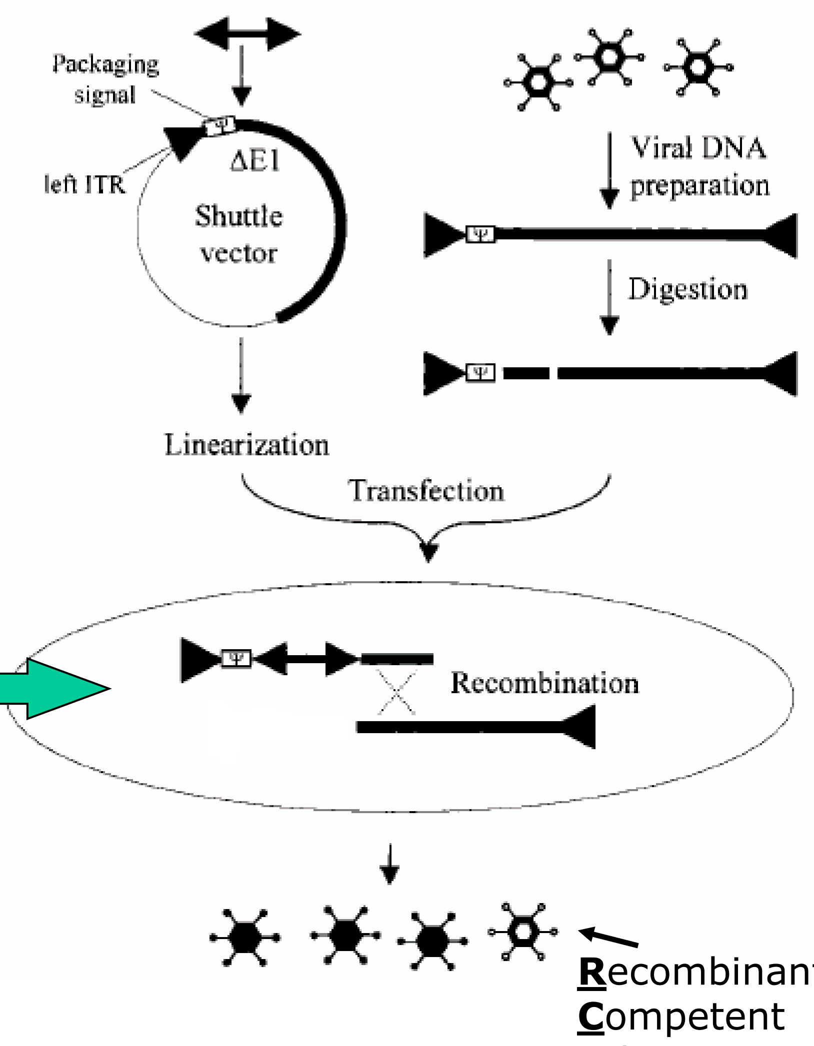


Adenoviral vector's (AdV) infection of HEK 293 cells



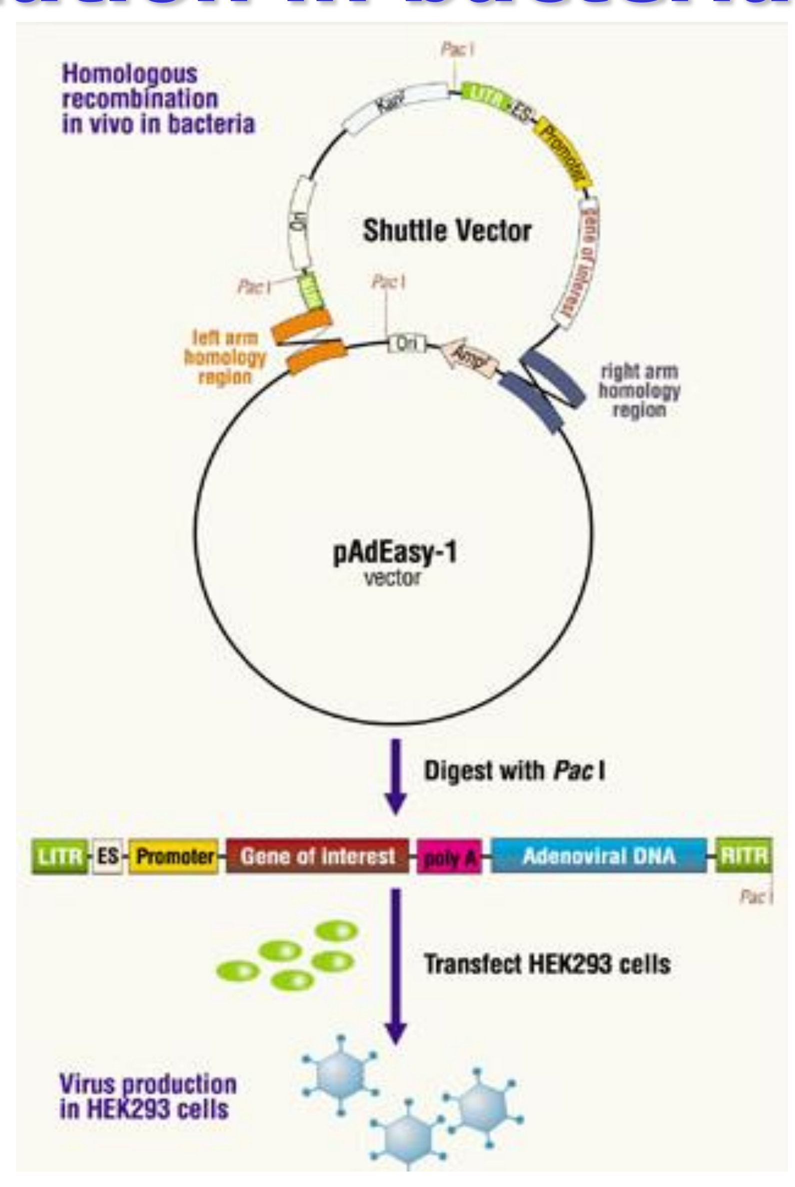
AdV plasmid construction I: homologous recombination in HEK 293

- Shuttle vector (containing transgene and packing site) and Ad-genome with overlapping fragments
- E1 region in Ad-genome digested with restrictase
- Spontaneous, homologous recombination in HEK293 $\Rightarrow$ AdV-genome
- AdV isolated from plaques used for further HEK 293 infections



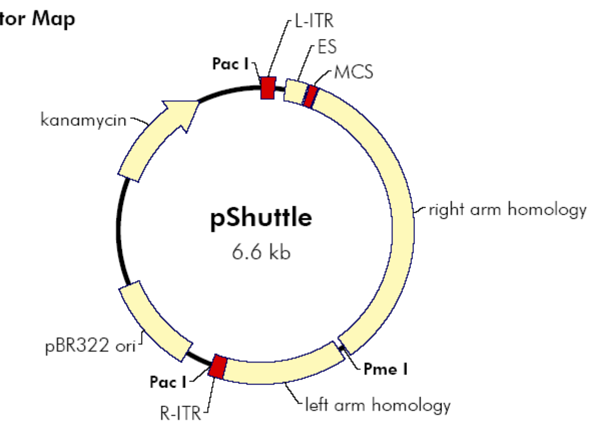
AdV plasmid construction II: homologous recombination in bacteria

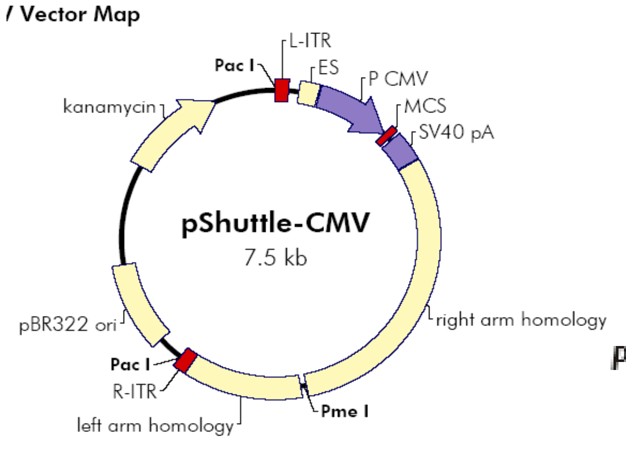
- Once constructed (via restriction digestions, ligations) the shuttle vector with transgene is linearized with *Pme* I and cotransformed into BJ5183 bacteria together with pAdEasy-1, the supercoiled viral DNA plasmid.
- Transformants are selected for kanamycin resistance, and recombinants are subsequently identified by restriction digestion.
- Once a recombinant is identified, it is produced in bulk using the recombination-deficient XL10-Gold® strain.
- Purified recombinant Ad plasmid DNA is digested with *Pac* I to expose its ITR and then used to transfect HEK293.



Cloning transgene into different shuttle vectors

MCS (multicloning site) of most shuttle vectors is flanked by promoter and poli-A signal to ensure proper expression cassette of transgene





'P-1 Vector Map

pShuttle-IRES-hrGFP-1

8.9 kb

L-ITR

Pac I

ES

P CMV

MCS

3x FLAG

IRES

kanamycin

hrGFP

pBR322 ori

SV40 pA

Pac I

R-ITR

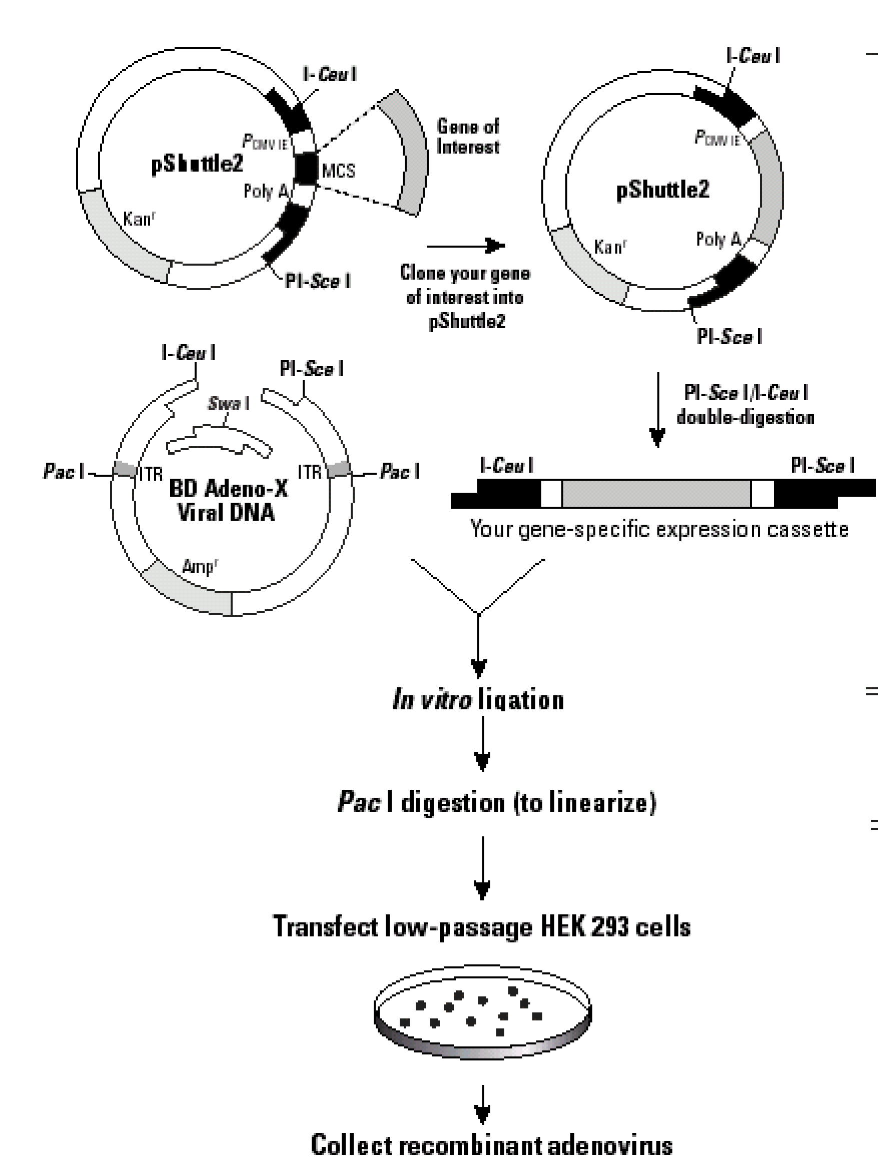
left arm homology

Pme I

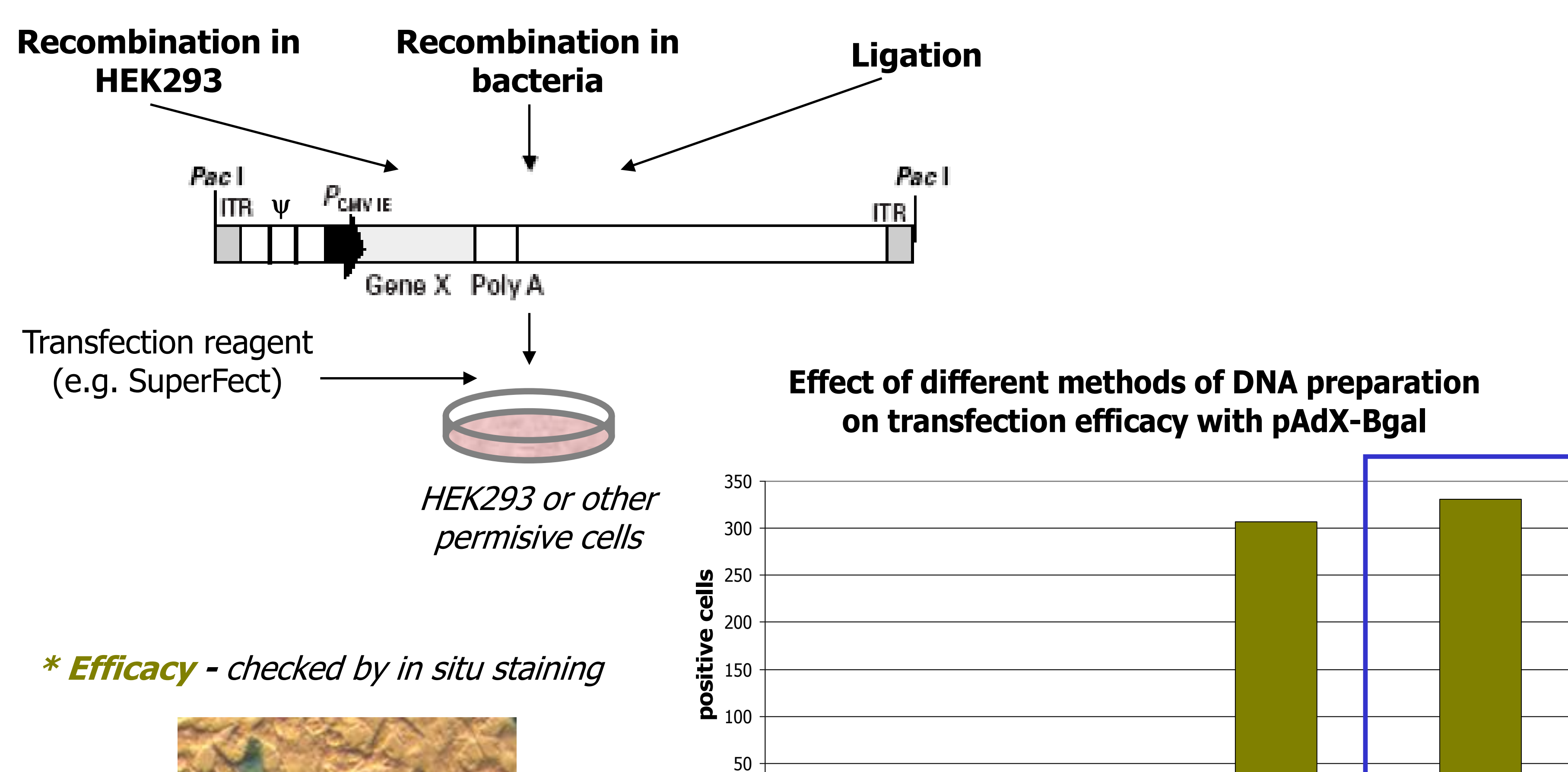
right arm homology

AdV plasmid construction III: ligation

- Cloning cDNA of gene of interest into pShuttle2 (plasmid contains MCS flanked by CMV promoter and polyA)
- Subcloning the expression cassette from pShuttle2 into pAdeno - double digestion of pShuttle2 and pAdeno with I-CeuI and PI-SceI
- Ligation of pAdeno with digested expression cassette
- Selection on ampicilin resistance, amplification of constructed AdV genome
- Linearization of adenoviral genome to expose ITRs (digestion with PacI)
- Transfection of permissive cell line (with E1 region) with linearized genome to produce capsids and pack AdV-DNA into them.



Production of AdV from constructed plasmid in permissive cells



Cells for production of AdV (permissive cells)

- HEK293 (human embryonic kidney) the most often used, stably transfected with E1 vector
  * adherent [optimistic version....] (293A, 293H)
    ~ suitable for plaque and CPE based assays
  * not adherent (293F, 293H)
    ~ allow for production of higher titers of viruses

- GH329 (HeLa derived cells) - postulated lower recombination rate

- PER.C6 (human embryonic retinoblast) – similar to 293 but with lower recombination rate

- HER 911 (human embryonic retinoblast) – similar to 293, but better for plaque forming assay, gives higher titers of Ad (up to 3 fold)

- A549 (human lung carcinoma) – very low level of recombination

Production of AdV from constructed plasmid in permissive cells

Recombination in HEK293

Recombination in bacteria

Ligation

Pac I

ITR ψ $P_{CMV\ IE}$

Gene X Poly A

Pac I

ITR

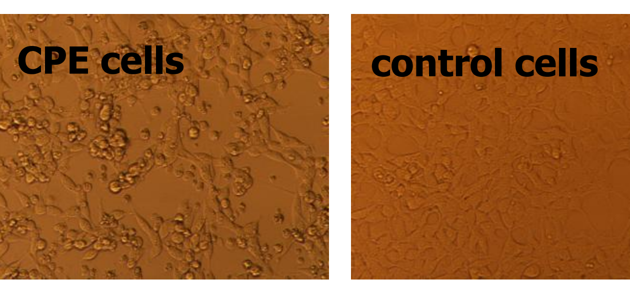
Transfection reagent (e.g. SuperFect)

AdV propagation monitored either by reporter protein expression…

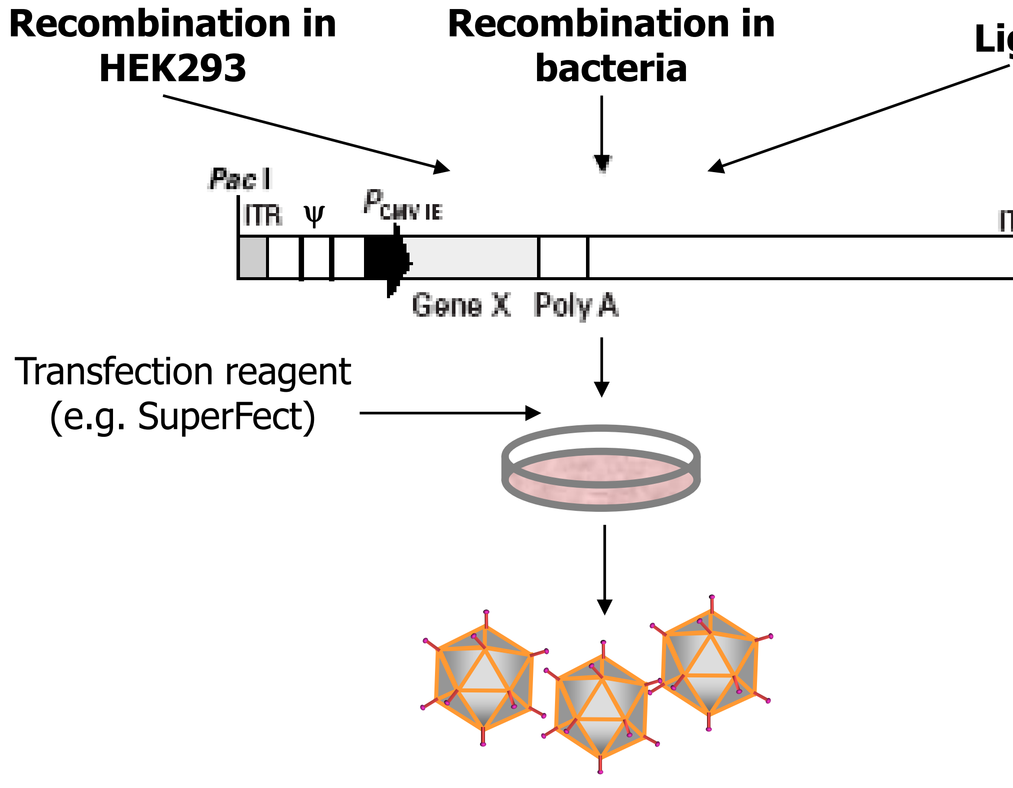
Day 1 Day 3 Day 7

… or cythopatic effect (CPE) progression.

Harvesting primary stocks (from 1st passage) of AdV from infected cell cultures



Production of AdV from constructed plasmid in permissive cells

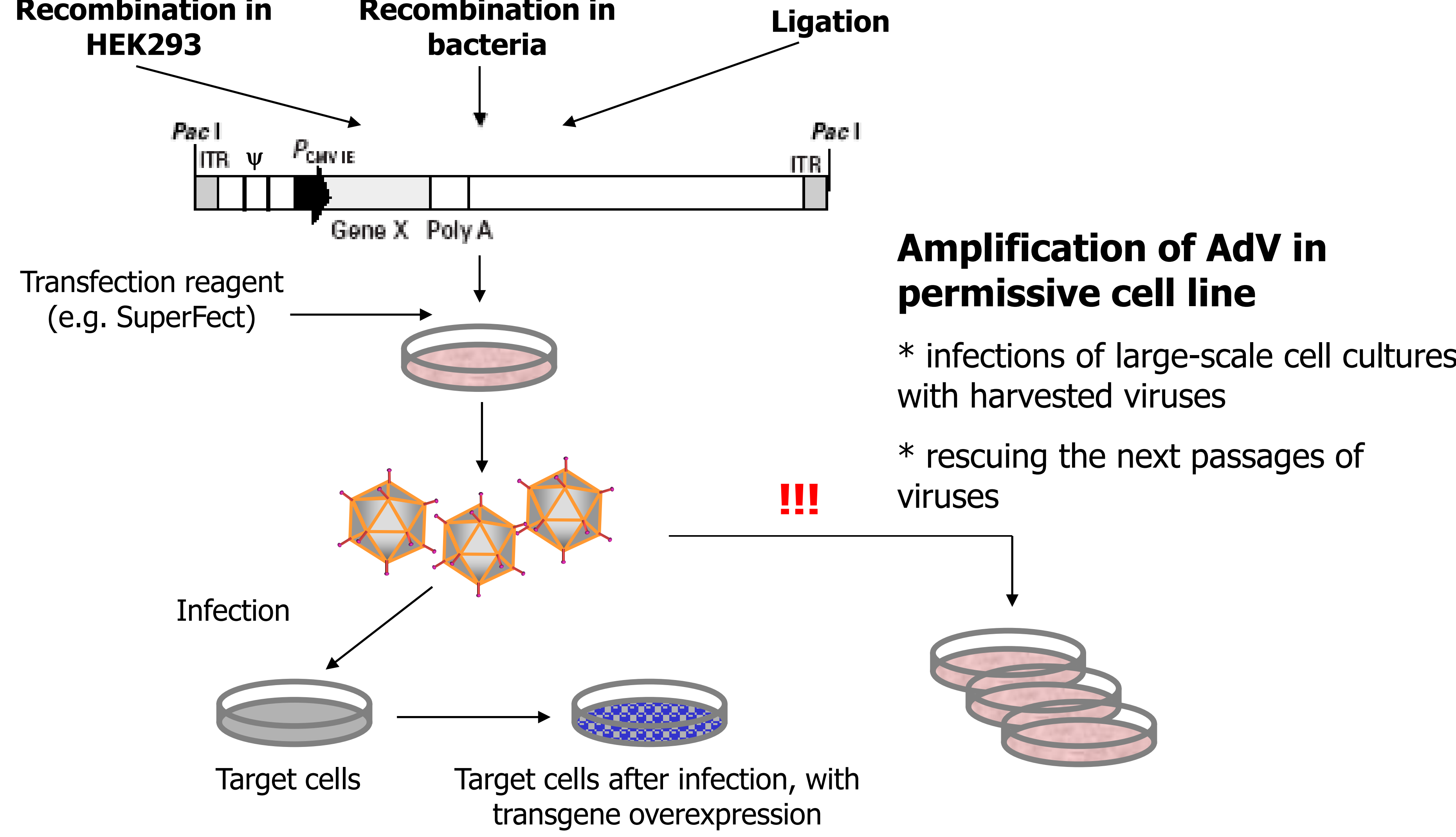


Harvesting primary stocks (from 1st passage) of AdV from infected cell cultures

Methods of isolation of AdV from infected permissive cells:

- chemical lysis of cells – can destroy capsids
- harvesting AdV from medium, after cell lysis – low concentration of AdV
- harvesting AdV from cells by freeze-thaw method, before cell lysis

Production of AdV from constructed plasmid in permissive cells

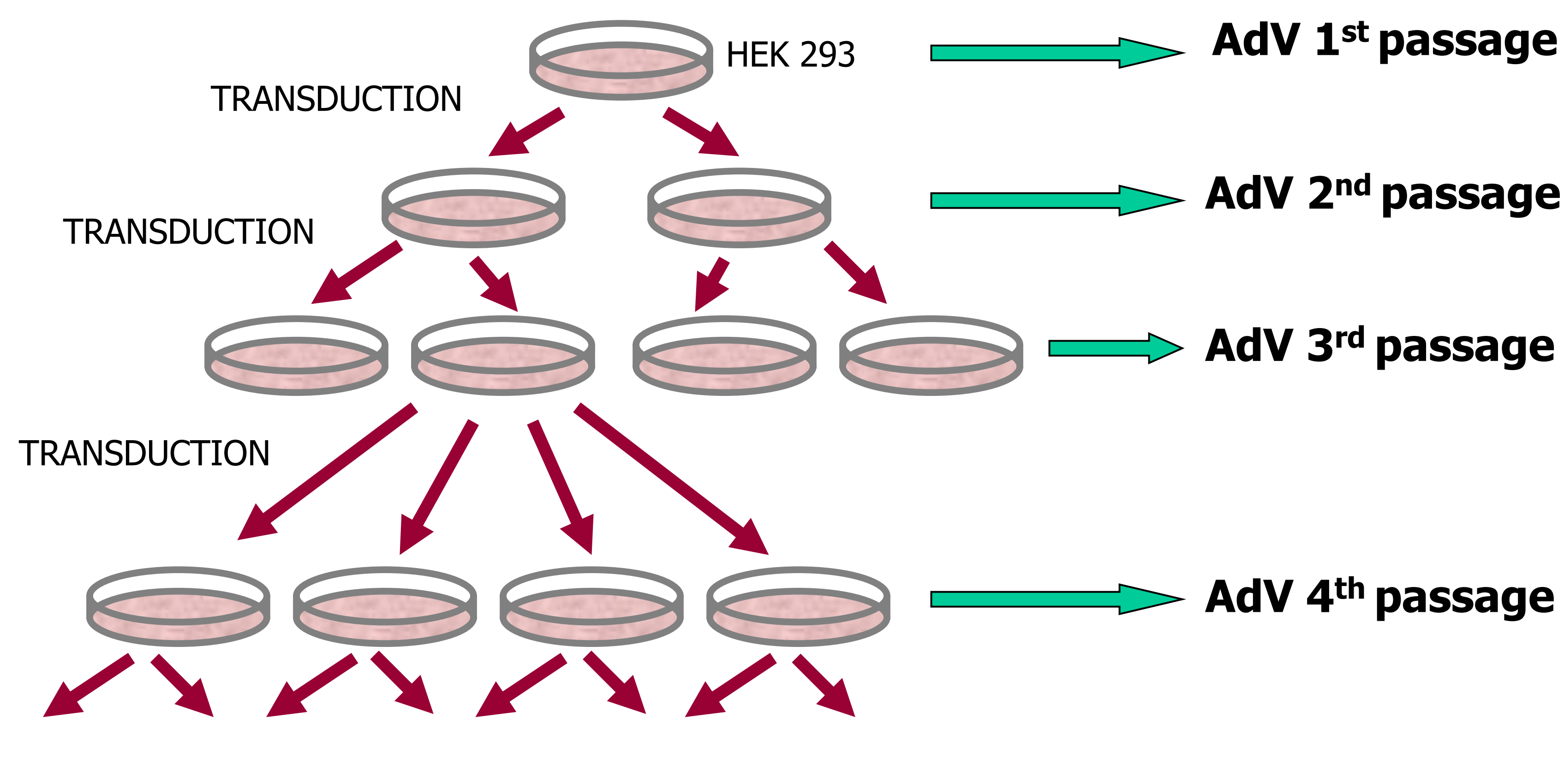


Amplification of AdV in permissive cell line

* infections of large-scale cell cultures with harvested viruses

* rescuing the next passages of viruses

Amplification of AdV in permissive cells



...

Amplification - cheap, fast, easy method to:

- Increase amount of vector lysate
- Increase of vector titer

Amplification of AdV in permissive cells

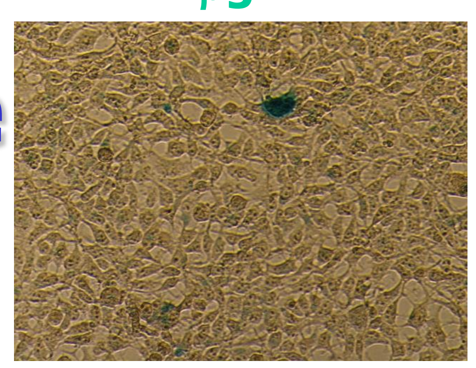
titer [IU/ml]

1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
1,00E+00

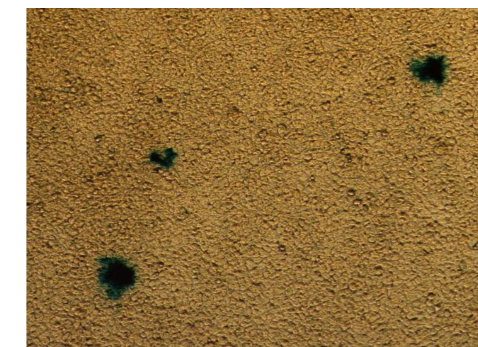
1' 2' 3' 4' 5' 6'

passages of vector

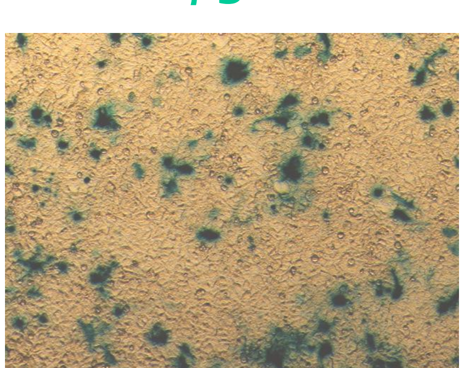
Ad-βgal 1st



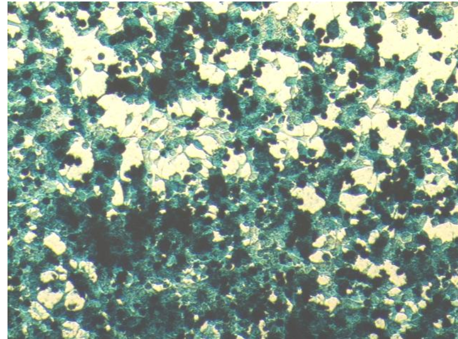
Ad-βgal 2nd



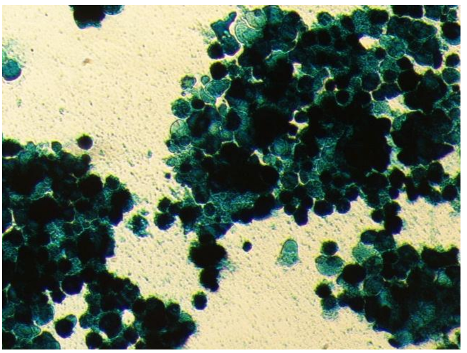
Ad-βgal 3rd



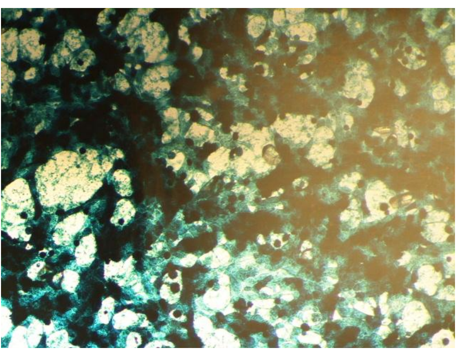
Ad-βgal 4th



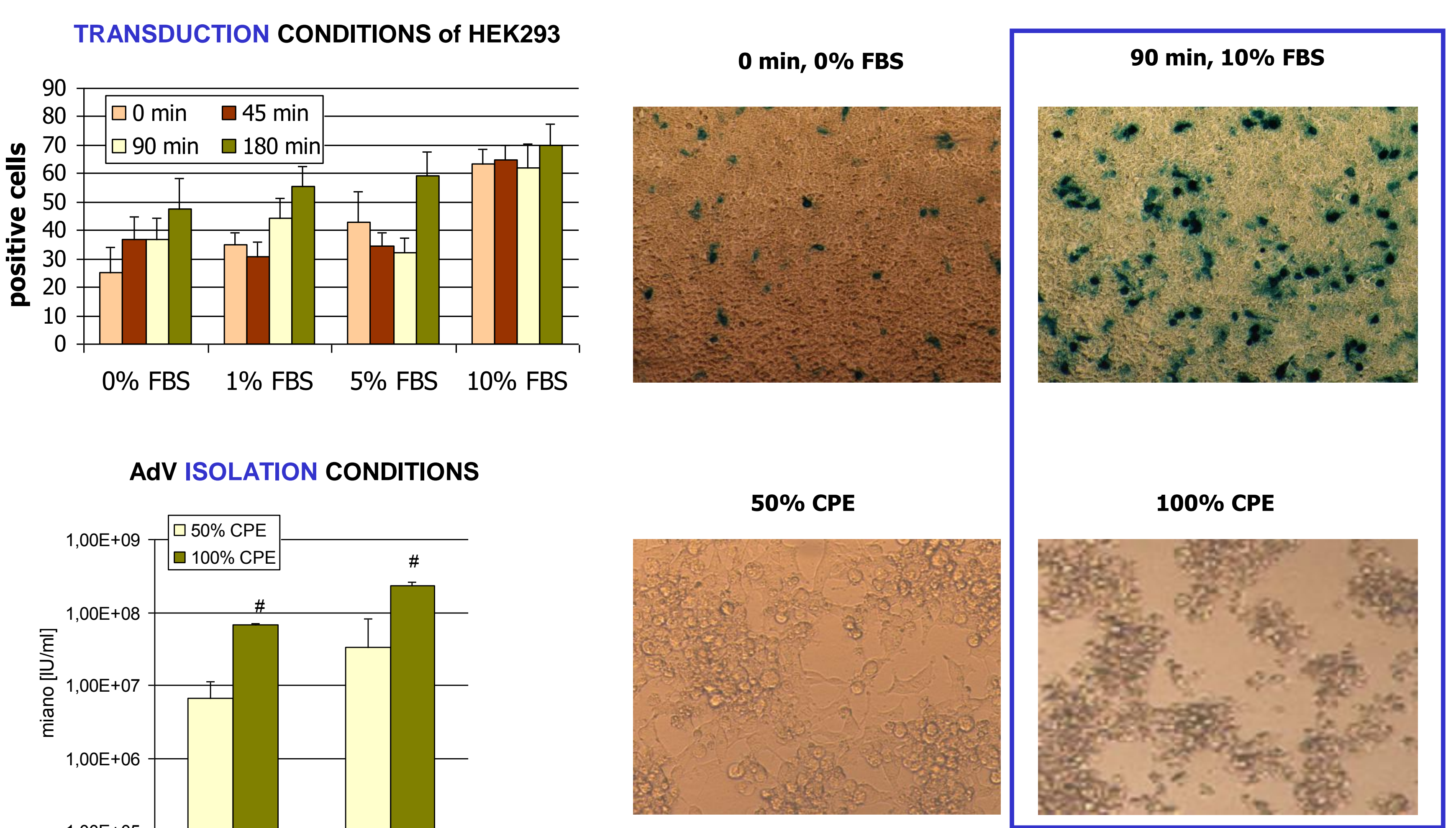
Ad-βgal 5th



Ad-βgal 6th



Amplification of AdV in permissive cells - improving propagation



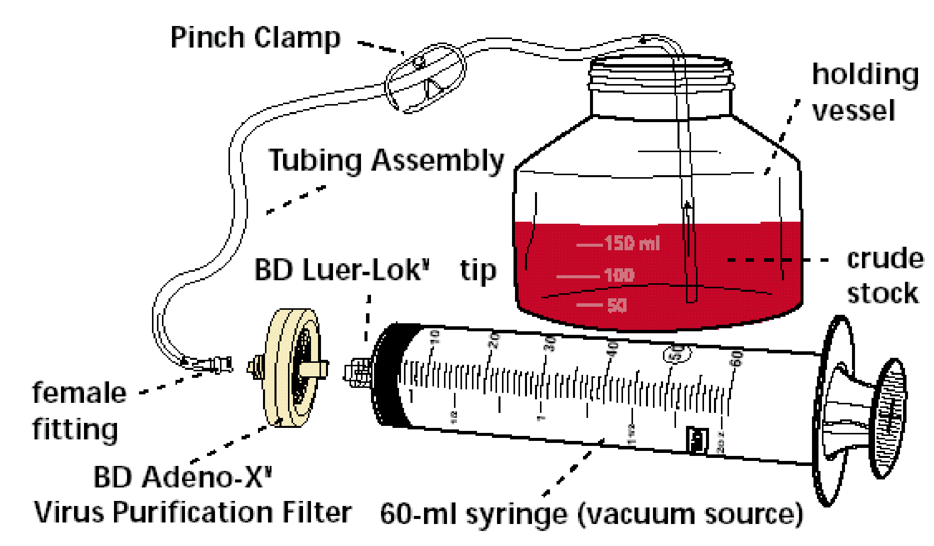
M. Kozakowska et al. Biotechnologia, 3(78) 2007

Purification of AdV*

- **Crude lysates** (after freeze-thaw isolation, without purification, contain PBS + AdV + proteins from HEK293) can be used for **in vitro** experiments
  - High AdV titer -> **small volume** (~ 1 ul) of lysates used -> small contamination with proteins released from lysated HEK 293 cells

- **Purified lysates** can be used for **in vivo** experiments
  - **Large volumes** (~ 200 ul) used -> large contamination with proteins released from lysated HEK 293 cells

- Purification methods:
  - **CsCl** ultracentifiguration: effective but tricky
  - **Chromatographically**: capsids have affinity to a membrane -> elution purified and concentrated AdV with elution buffer



Titration of AdV

- **Physico-chemical methods**
  - Based on direct measurements of concentration of AdV capsids or AdV genome, **without assessing their functionality**
  - Easy, fast and objective
    - OD 260 assay
    - Real-time PCR

  } **VP/ml (Vector Particles / ml)**

- **Biological methods**
  - Based on infectious properties of AdV – involve infection of permissive cells with serial dilution of AdV
  - Titer calculation is based on visual detection of CPE positive cells, transgene-positive cells or cells producing capsid protein – **asses only fully functional AdV particles**
  - Less objective, more time-consuming
    - Plaque formation assay
    - End Point dilution assay
    - Reporter gene assay
    - Rapid Titer Elisa ™

  } **IU/ml (Infectious Units / ml)**

OD_{260} assay

Calculate viral titer and purity

viral titer (opu/ml) = OD_{260} x viral dilution x 1.1×10^{12}

opu = opticle particle unit

Note: Because opticle particle units (opu) and plaque forming units (pfu) define different properties, these measurements cannot be directly compared.

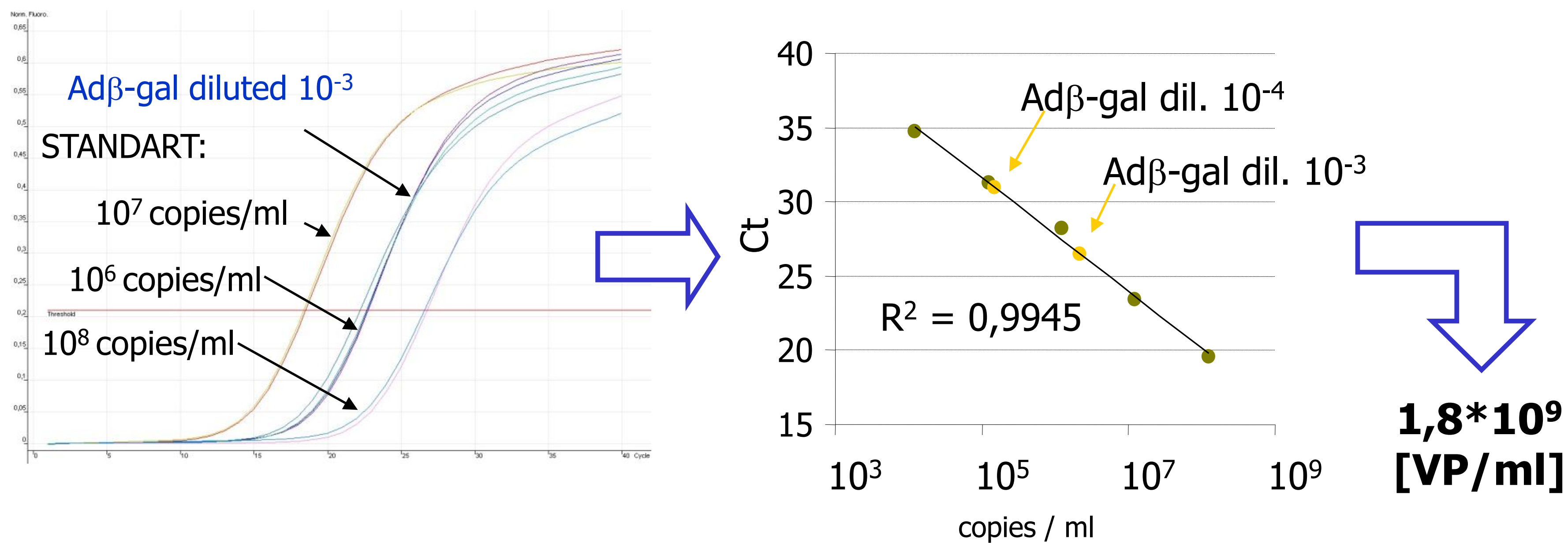
purity = OD_{260}/OD_{280} (typically ~1.2–1.3 after CsCl purification)

+ Not sensitive to human bias
+ Cheap, fast, easy
+ Reproducible results
+ Does not depend on infection conditions

- Not suitable for crude lysates, as debris will interfere with readings
- Does not distinguish between fully functional and impaired AdV (empty capsids) - overestimates functional titer

Real-Time PCR

- Based **quantitative relationship** between the amount of starting target DNA and the amount of amplification product during the exponential phase of a cycling program
- Involves **standard curve** calculation of DNA copies/ml.
- Can be measured directly from crude lyastes



Functional titer of Adβ-gal used for Real-Time measurements: **1*10^8 [IU/ml]**

Real-Time PCR

+ Not sensitive to human bias
+ Fast and easy
+ Reproducible results
+ Does not depend on infection conditions

- Does not distinguish between fully functional and impaired AdV (DNA which has not been packed yet) – overestimates functional titer ~ 10 times
- Primers for every transgene or AdV genome should be designed
- Standard curve is needed

Plaque formation assay

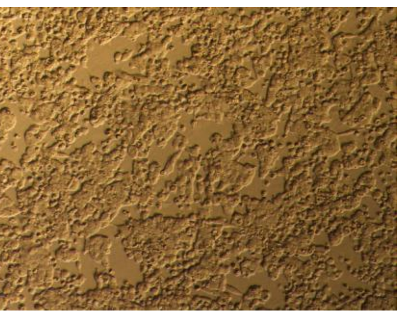
Visual detection of cytopathic cells

1. Prepare serial dilutions
2. Infect cells HEK293 cells on 6 wells plates
3. Incubate for 1,5 h
4. Cover wells with 2% agarose solution
5. Wait until plaques are visible (7–10 days)
6. Count the titer basing on visual detection of positive cells (plaques), assuming, that 1 plaque comes from 1 AdV particle

[IU/ml] = # plaques / (d * V)

IU – Infectious unit
d – dilution factor
V – volume of diluted viruses added per well

control


10^{-5} diluted

10^{-10} diluted

10^{-11} diluted

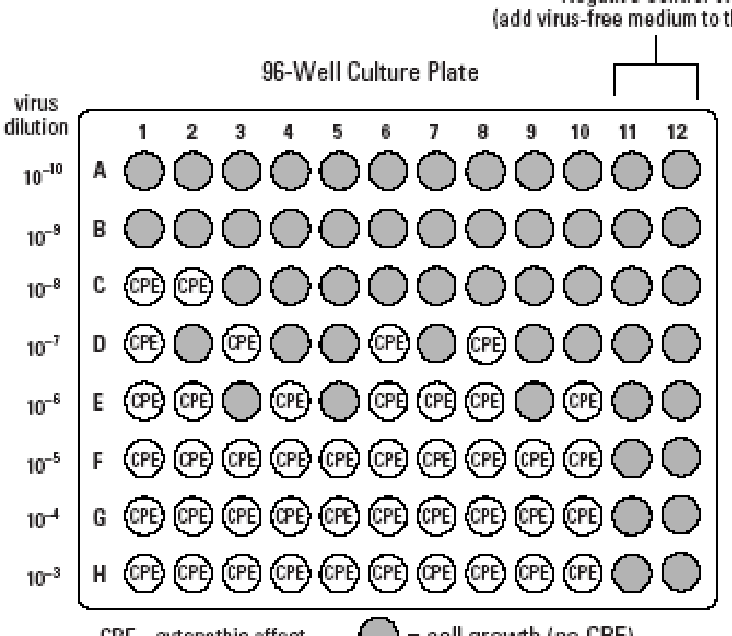
Plaque formation assay

\+ Cheap
\+ Detects positive (successfully infected) cells -> functional AdV

\- Very tricky and difficult to perform properly
\- Irreproducible results
\- Sensitive to human bias
\- Time consuming
\- Depends on infection conditions

End-point dilution assay

1. Prepare serial dilutions
2. Infect HEK cells on 96-plate
3. Wait 10-14 days until CPE will be visible
4. Count the titer basing on visual detection of CPE positive wells when following conditions are fulfilled
   - control wells mustn't show visible CPE
   - wells infected with the least diluted AdV are all CPE positive
   - wells infected with the most diluted AdV are all CPE negative



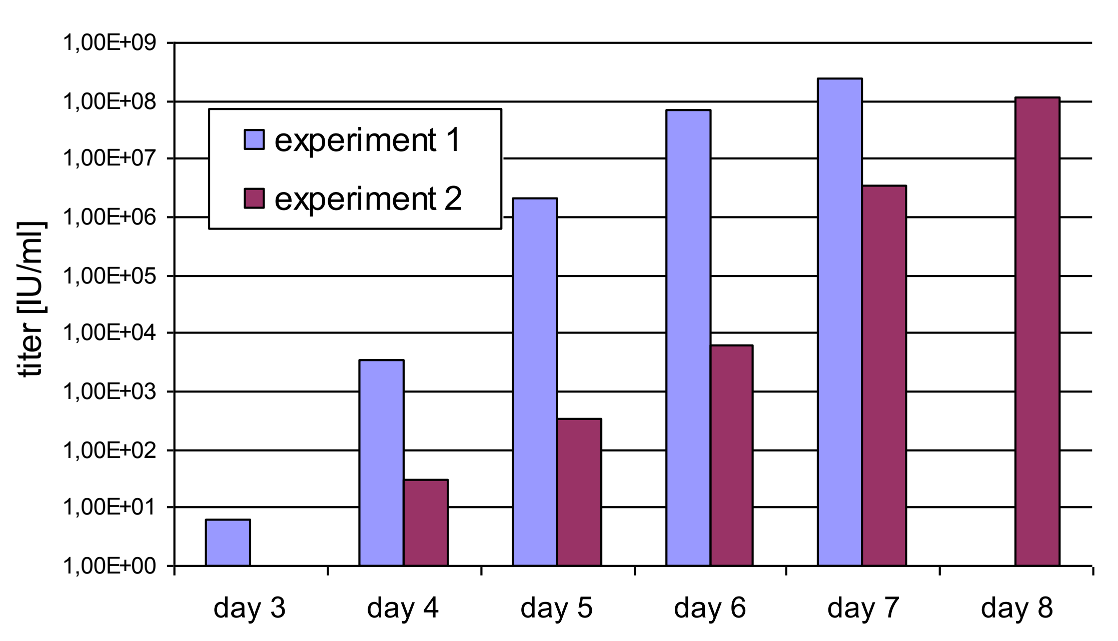
Dilution	Fraction of CPE-positive wells
10^{-10}	$0 \div 10 = 0$
10^{-9}	$0 \div 10 = 0$
10^{-8}	$2 \div 10 = 0.2$
10^{-7}	$4 \div 10 = 0.4$
10^{-6}	$7 \div 10 = 0.7$
10^{-5}	$10 \div 10 = 1$
10^{-4}	$10 \div 10 = 1$
10^{-3}	$10 \div 10 = 1$

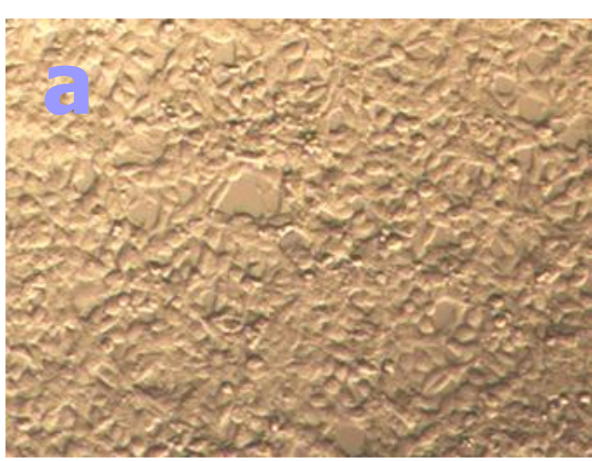
8. Calculate Viral Titer

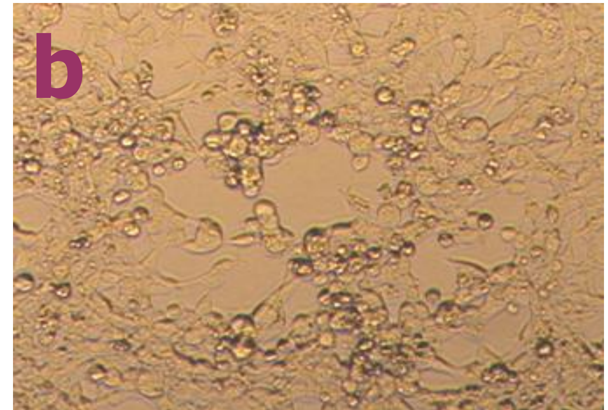
   Titer (pfu/ml) = $10^{(x + 0.8)}$

   x = the sum of the fractions of CPE-positive wells

End-point dilution assay



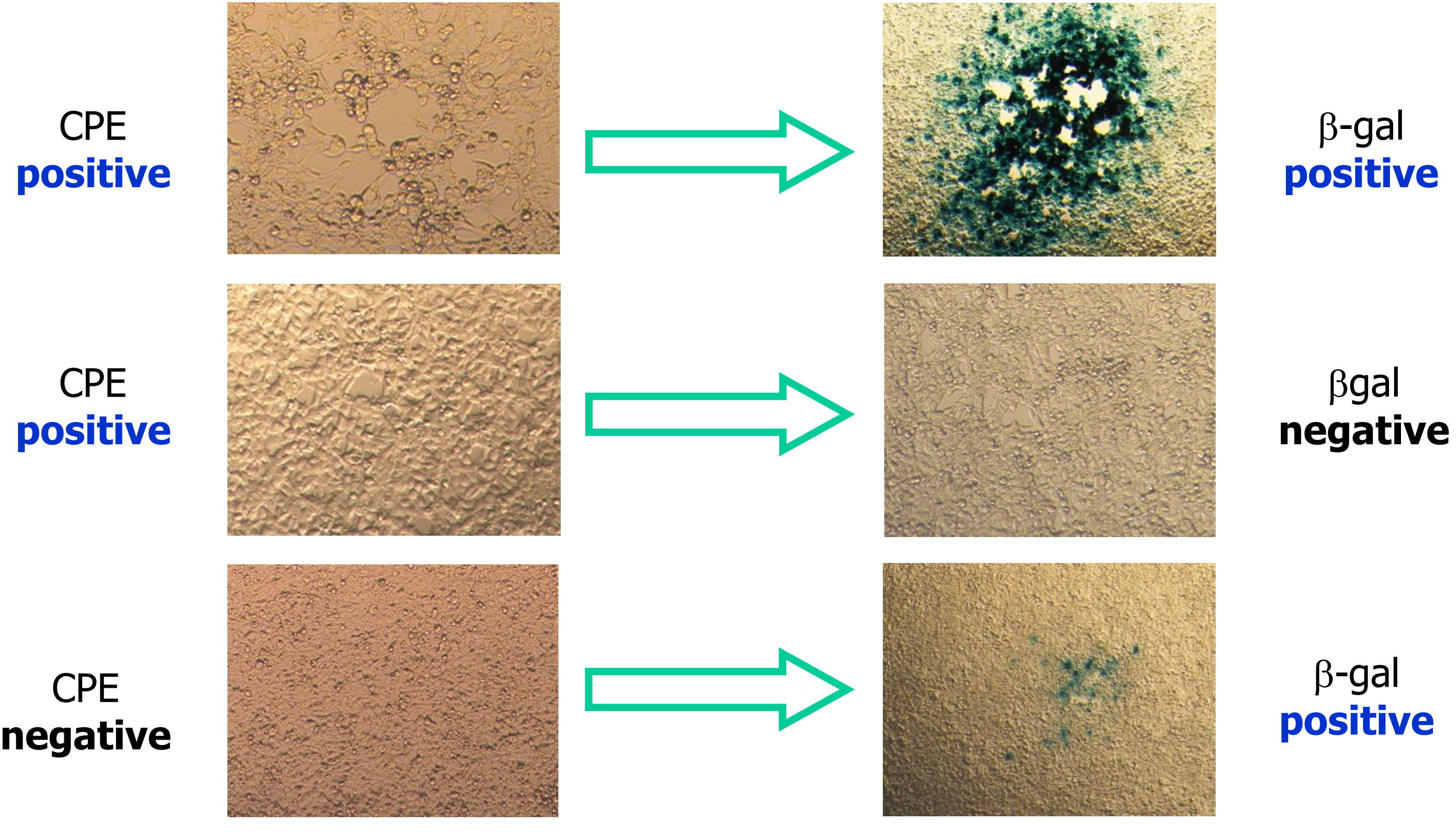




Experiment 1 – wells as on picture **a** recognised as CPE positive

Experiment 2 – wells as on picture **b** recognised as CPE positive

End-point dilution assay



End-point dilution assay

+ Cheap
+ Detects positive (successfully infected) cells -> functional AdV

- Very tricky and difficult to perform properly
- Irreproducible results
- Extremely sensitive to human bias
- Time consuming
- Gives false positive and false negative results
- Depends on infectious conditions

Reporter protein assay

1. Prepare serial dilutions and infect HEK cells on 96-plate
2. visual detection of positive cells after 48 hrs - β-gal *in situ* staining or microscopic observation of expressing fluorescent protein cells
3. Calculation of positive cells & calculation of titer [IU/ml] basing on assumption that 1 AdV infects 1 cell

Adβ-gal 1′ Adβ-gal 2′ Adβ-gal 3′ Adβ-gal 4′ Adβ-gal 5′ Adβ-gal 6′

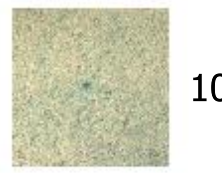
10^{-1}

10^{-2}

10^{-3}

10^{-4}

10^{-5}



10^{-7}

[IU/ml] = # positive cells / (d * V)

IU – Infectious unit
d – dilution factor
V – volume of diluted viruses added per well

Reporter protein assay

+ Cheap, easy and relatively fast (2 days)
+ Reproducible results
+ Detects positive (successfully infected) cells -> functional AdV

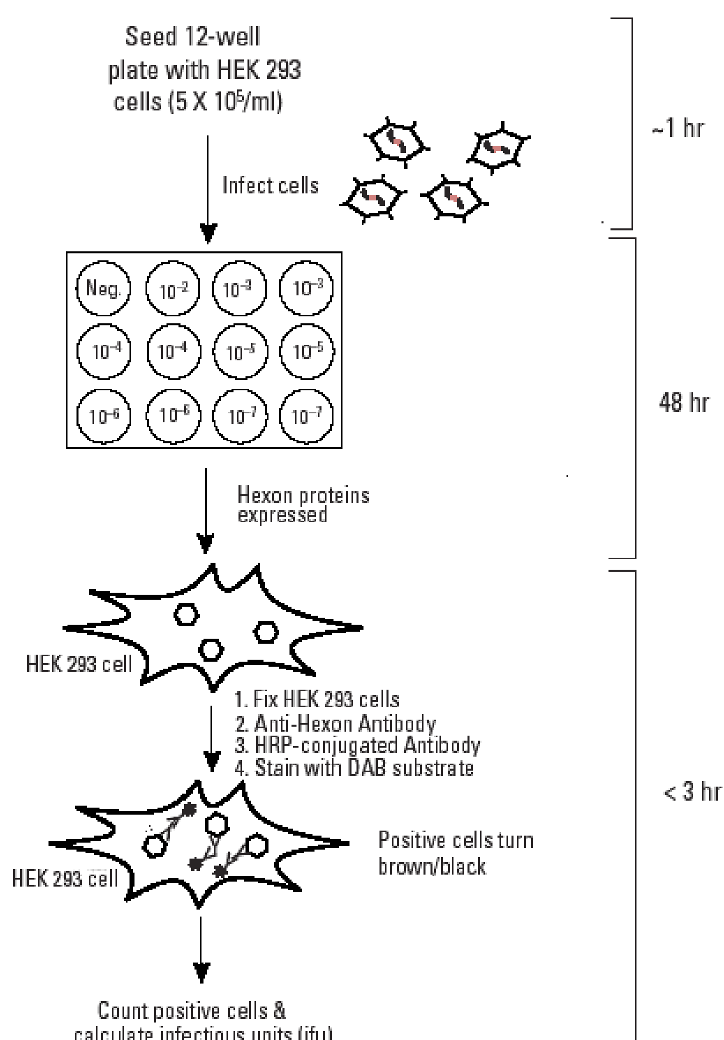
- Visual detection - sensitive to human bias
- Depends on infectious conditions
- Useful only for reporter proteins

Rapid Titer Elisa ™

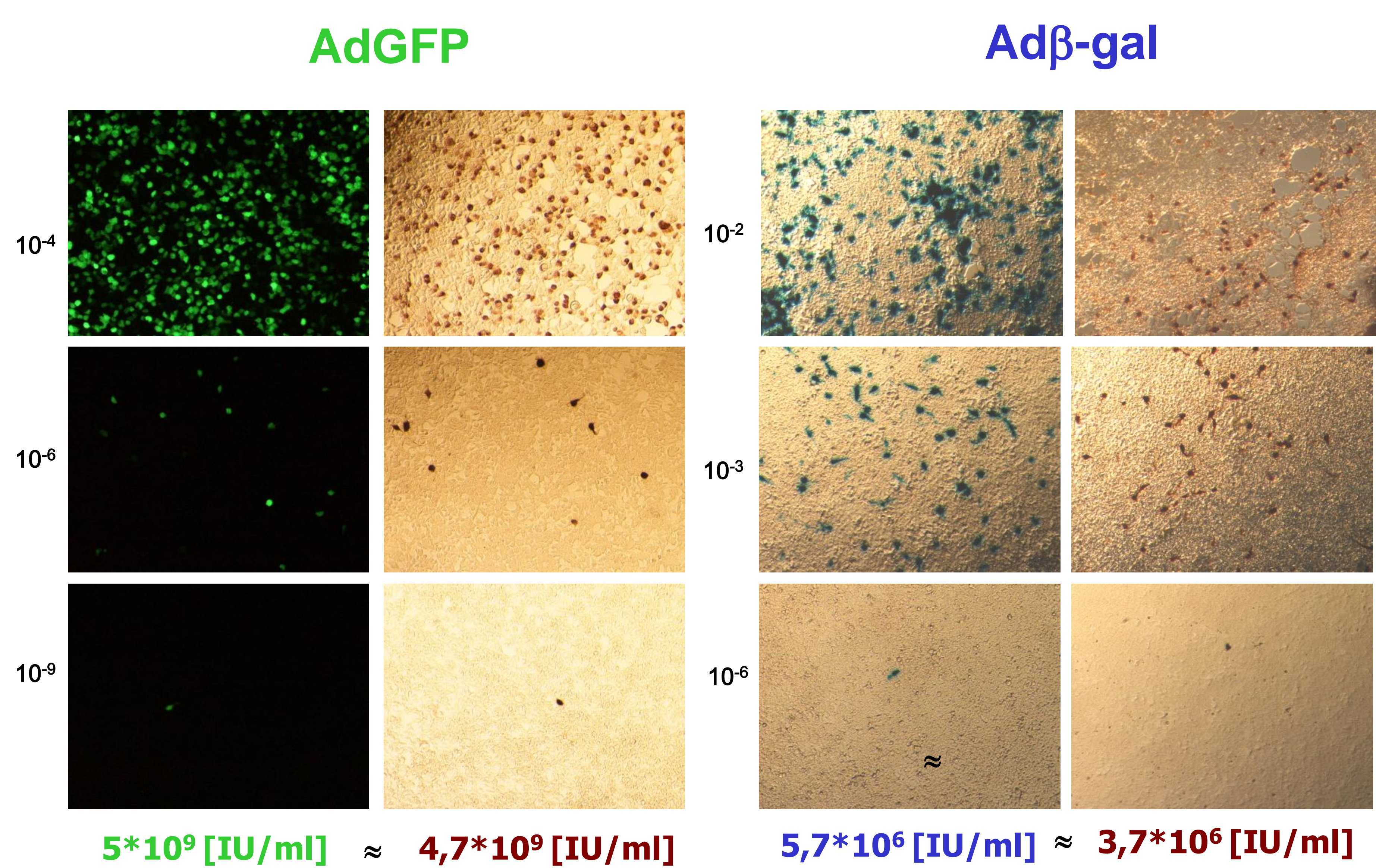
1. Prepare serial dilutions and infect HEK cells on 96-plate
2. ELISA on viral protein of capsid (hexon) – visual detection of positive cells (infected with AdV, producing viral progeny)
3. Calculation of positive cells & calculation of titer [IU/ml] basing on assumption that 1 AdV infects 1 cell

[IU/ml] = # positive cells / (d * V)

IU – Infectious unit
d – dilution factor
V – volume of diluted viruses added per well



Rapid Titer Elisa ™



Rapid Titer Elisa ™

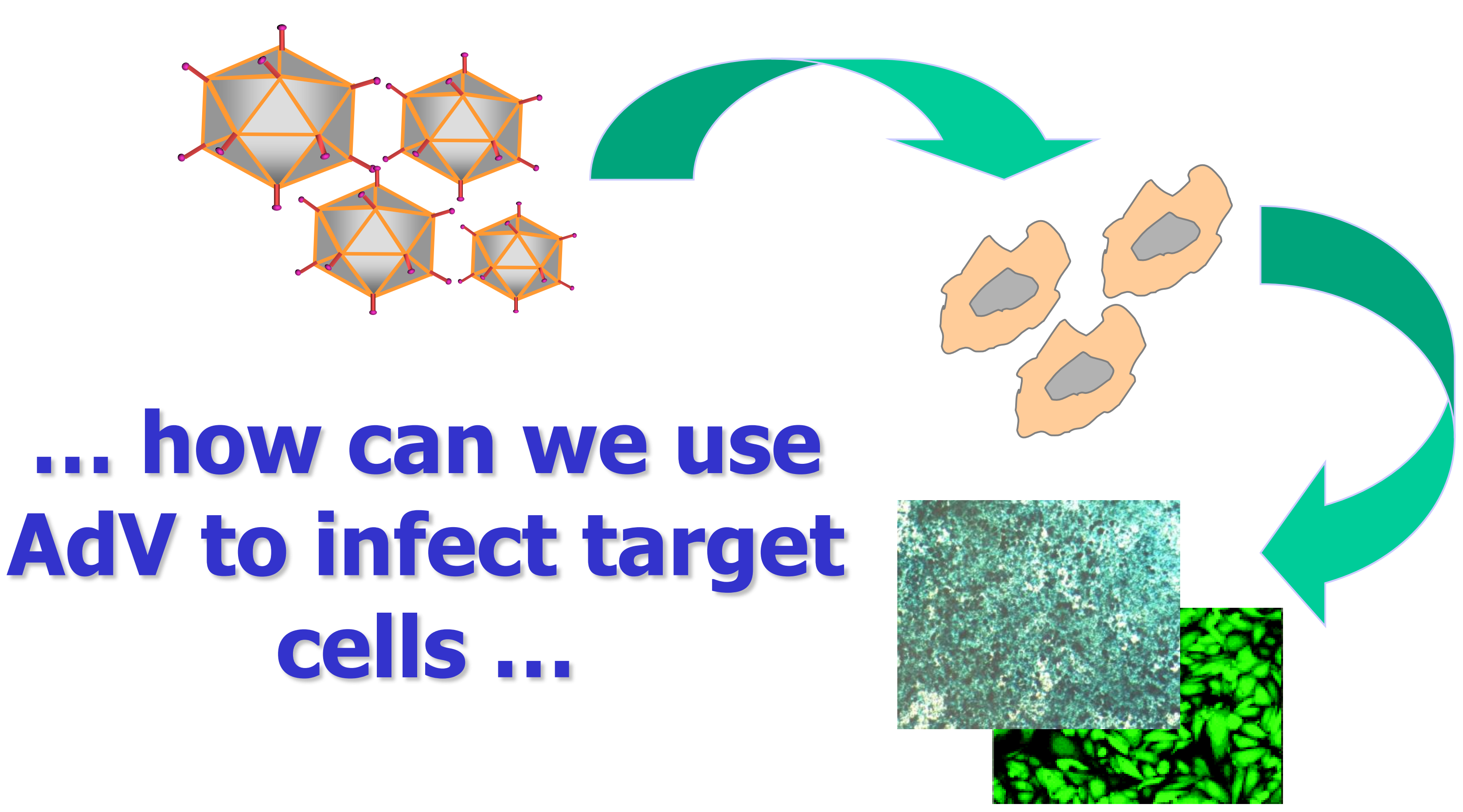
+ Easy, relatively fast (2 days)
+ Detects positive (successfully infected) cells -> functional AdV
+ Reproducible results
+ Correlates with titer assessed by reporter gene assay

- Sensitive to human bias
- Depends on infectious conditions

Titration of AdV

- Results from different titration methods can be different from each other.
- Physico-chemical methods give higher titer than biological methods because they detect not fully functional AdV as well
- Biological methods are less objective than physico-chemical methods and depend on infectious conditions, but they detect only fully-functional AdV

Production completed, so…



… how can we use AdV to infect target cells …

… to have transgene overexpression?

Calculation of amount of AdV needed

- **M.O.I.** = multiplicity of infection **[IU/cell]**
- Proper MOI (providing high infection efficacy and no decrease in viability of tranducted cells) should be establish for cell line we want to modify.
- Calculations:

$1*10^5$ cells in a well
MOI = 50

} $5*10^6$ IU / well

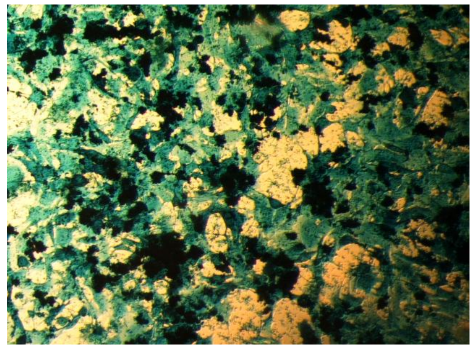
Titer AdV = $5*10^9$ IU/ml

$5*10^9$ IU - 1 ml
$5*10^6$ IU - x ml

$x = 5*10^6 / 5*10^9 = 0,001$ ml = 1 ul

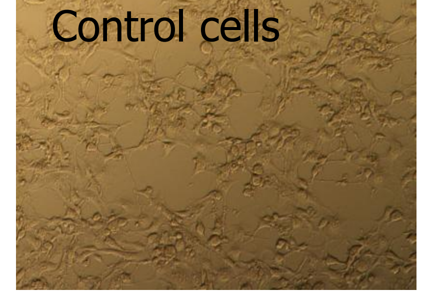
- To infect $1*10^5$ cells with MOI = 50, using AdV = $5*10^9$ IU/ml we have to add 1 ul of lysate per well

HUVEC cells



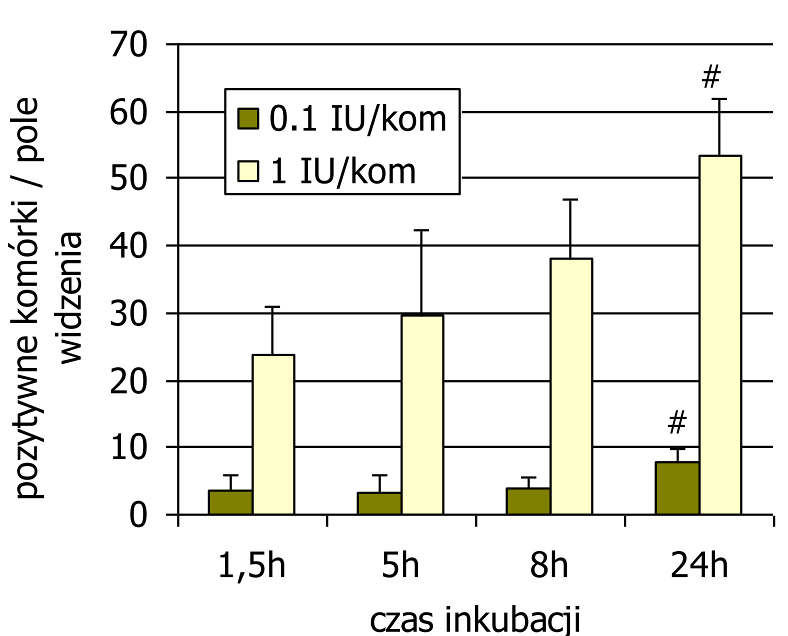
100 IU/kom

Efficacy of infection depends on amount of vector...

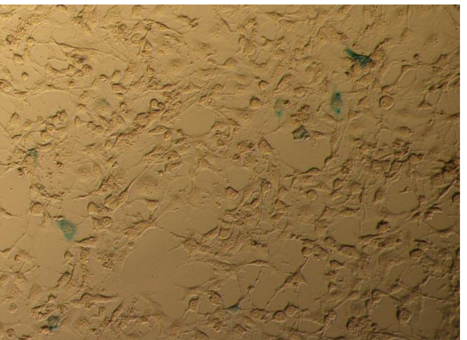


10 IU/kom

1 IU/kom



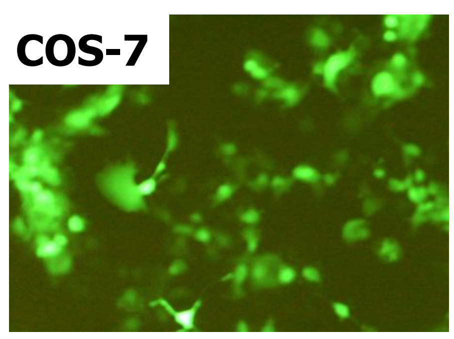
- $p < 0.05$ vs 1,5h

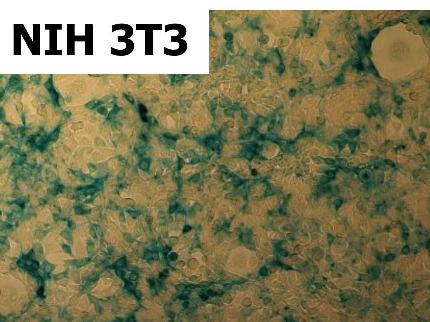


0,1 IU/kom

Transduction of different cell lines

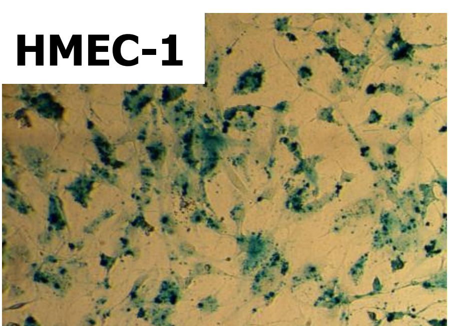
10 IU/cell
8 h
100%

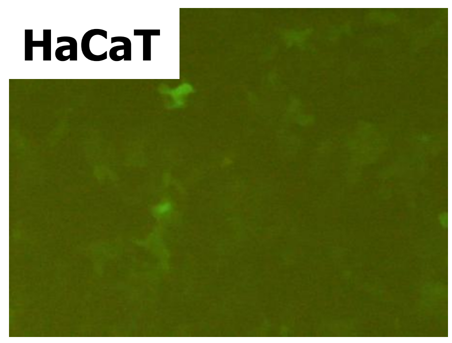




10 IU/cell
24 h
90%

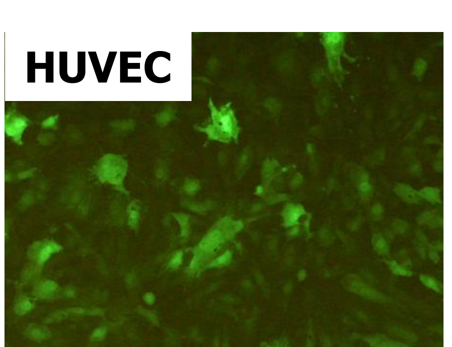
10 IU/cell
24 h
100%

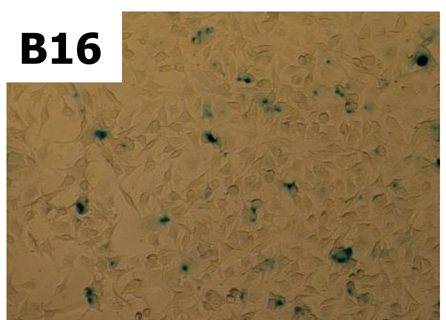




10 IU/cell
24 h
90%

10 IU/cell
24 h
100%





10 IU/cell
24 h
30%

M. Kozakowska et al. Biotechnologia, 3(78) 2007

Thank you for your attention and good luck!