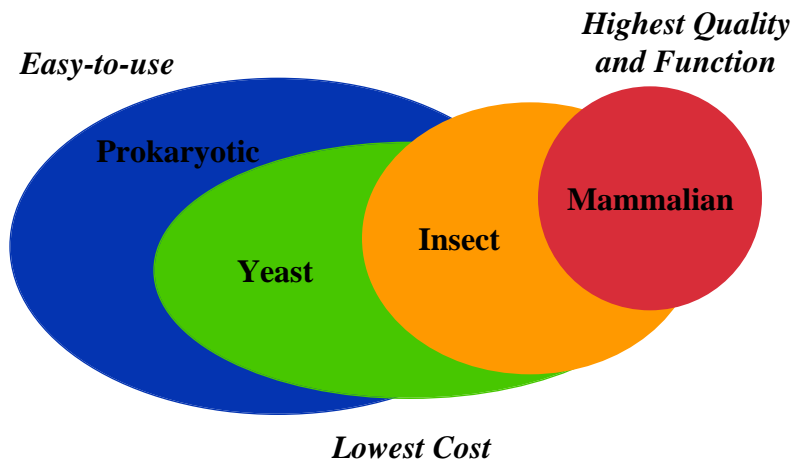


## Gene Expression Systems



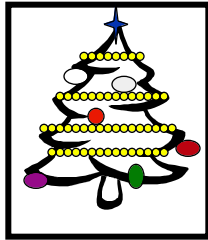
 **Invitrogen**  
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## Eukaryotic Alternatives to *E. coli*

- ***E. coli* is typically the first choice for expression of recombinant proteins**
- **However, there are often reasons that a eukaryotic host is required**
  - Posttranslational modifications
  - Protein folding
  - *In vivo* functional studies

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## Post-translational modifications



Prokaryotes

Yeast  
(lower eukaryote)



Insects  
(higher eukaryote)



Mouse cell line

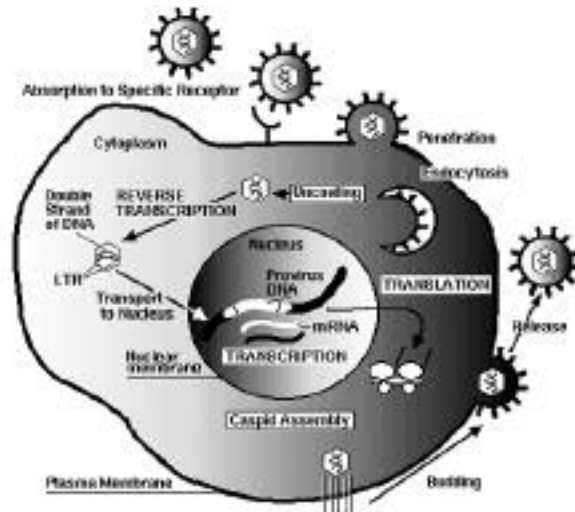


## Harnessing Nature's Solutions to Gene Expression Needs

- Eukaryotic Expression Systems

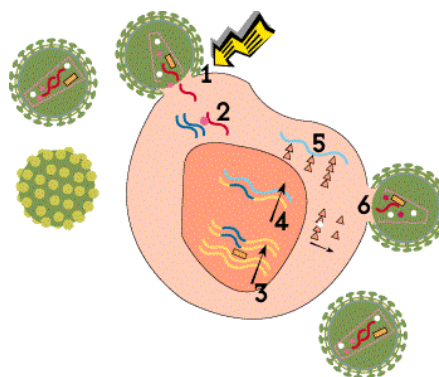


## Retroviruses targetting mammalian cells



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## The HIV Life Cycle

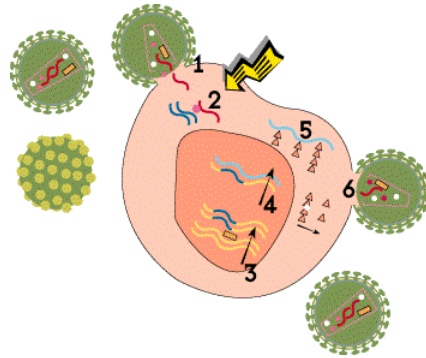


### Step 1: Binding

A virus consists of an outer envelope of protein, fat and sugar wrapped around a set of genes (in the case of HIV, genetic information is carried as RNA instead of DNA) and special enzymes. HIV has proteins on its envelope that are strongly attracted to the CD4+ surface receptor on the outside of the T4-cell. When HIV binds to a CD4+ surface receptor, it activates other proteins on the cell's surface, allowing the HIV envelope to fuse to the outside of the cell.

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## The HIV Life Cycle



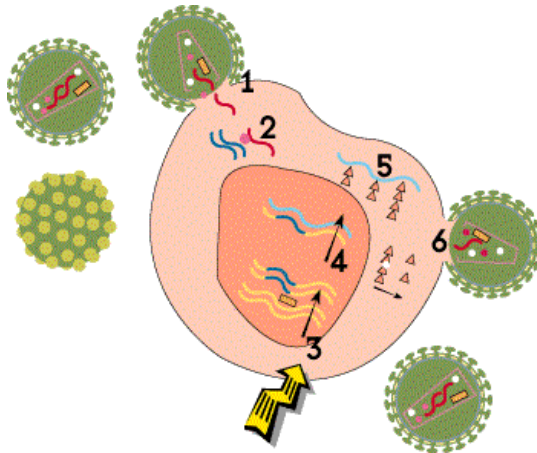
### Step 2: Reverse Transcription

HIV's genes are carried in two strands of RNA, while the genetic material of human cells is found in DNA. In order for the virus to infect the cell, a process called "reverse transcription" makes a DNA copy of the virus's RNA.

After the binding process, the viral capsid (the inside of the virus which contains the RNA and important enzymes) is released into the host cell. A viral enzyme called reverse transcriptase makes a DNA copy of the RNA. This new DNA is called "proviral DNA."



## HIV Life Cycle

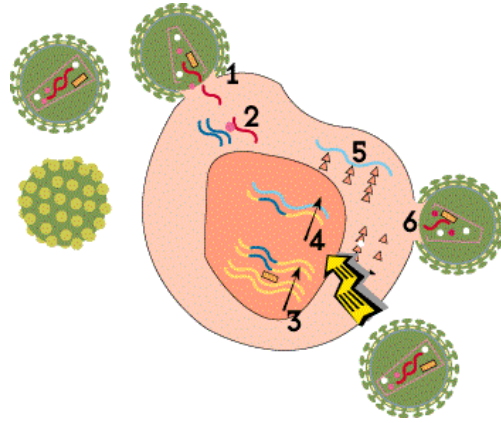


### Step 3: Integration

The HIV DNA is then carried to the cell's nucleus (center), where the cell's DNA is kept. Then, another viral enzyme called integrase hides the proviral DNA into the cell's DNA. Then, when the cell tries to make new proteins, it can accidentally make new HIVs. (



## The HIV Life Cycle



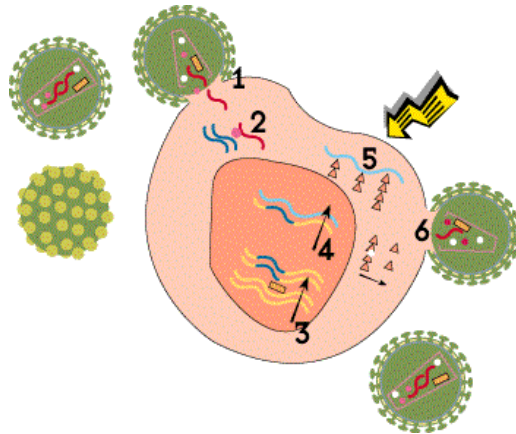
### Step 4: Transcription

Once HIV's genetic material is inside the cell's nucleus, it directs the cell to produce new HIV.

The strands of viral DNA in the nucleus separate, and special enzymes create a complementary strand of genetic material called messenger RNA or mRNA (instructions for making new HIV).



## The HIV Life Cycle



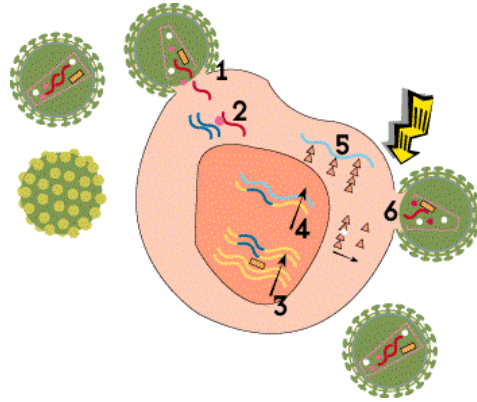
### Step 5: Translation

The mRNA carries instructions for making new viral proteins from the nucleus to a kind of workshop in the cell. Each section of the mRNA corresponds to a protein building block for making a part of HIV.

As each mRNA strand is processed, a corresponding string of proteins is made. This process continues until the mRNA strand has been transformed or "translated" into new viral proteins needed to make a new virus.



## The HIV Life Cycle



### Step 6: Viral Assembly

Finally, a new virus is assembled. Long strings of proteins are cut up by a viral enzyme called protease into smaller proteins. These proteins serve a variety of functions; some become structural elements of new HIV, while others become enzymes, such as reverse transcriptase. Once the new viral particles are assembled, they bud off the host cell, and create a new virus. This virus is then able to infect new cells. Each infected cell can produce a lot of new viruses.

## What have we learned.....



## Genes important for the lentivirus system



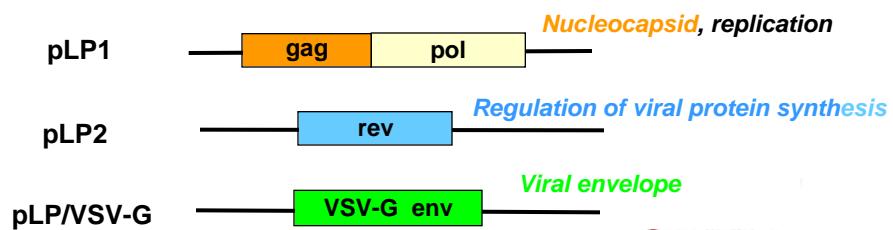
## Lentiviral Packaging Proteins



Retroviral **packaging proteins** (gag/pol, rev, and env) are supplied ***in trans***.

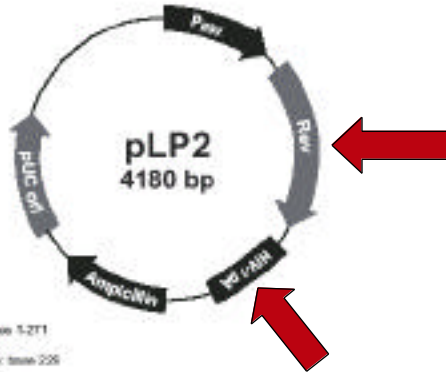
This provides two safety features:

- Genes coding viral proteins will not be incorporated into the RNA genome of the final viral particle
- System doesn't use the HIV envelope





# Lentivirus System

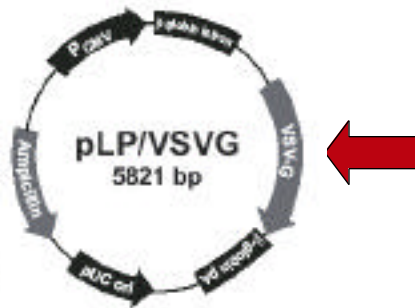
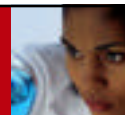


Comments for pLP2  
4180 nucleotides

RSV enhancer/promoter: bases 1-211  
TATA box: bases 233-257  
Transcription initiation site: base 226  
RSV UTR: bases 230-271  
HIV-1 RSV ORF: bases 281-341  
HIV-1 LTR polyadenylation signal: bases 650-671  
CMV promoter: bases 1916-2014  
Ampicillin (bla) resistance gene: bases 2335-2575  
pUC origin: bases 3025-3653



# Lentivirus System



Comments for pLP/VSVG  
5821 nucleotides

CMV promoter: bases 1-747  
TATA box: bases 646-651  
HIV-1 LTR: bases 660-1020  
VSVG glycoprotein (VSVG-gp): bases 1260-2281  
HIV-1 LTR polyadenylation signal: bases 3004-3180  
pUC origin: bases 3827-4643 (C)  
Ampicillin (bla) resistance gene: bases 4745-5625 (C)  
bla promoter: bases 5426-5724 (C)  
D-complementary strand



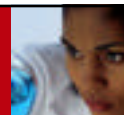
## Working with the Lentiviral Expression System



- Day 0: Plate  $5 \times 10^6$  293FT cells per 100mm plate (packaging cell line)
- Day 1: Transient transfection of 4 plasmids into a high-producing cell line (293FT)
- 3 packaging plasmids (pLP1, pLP2 & pLP/VSV-G)
  - 1 expression plasmid (pLenti6) with gene of interest (Gateway or d-TOPO cloning versions available)
- Day 2: Replace media
- Day 3-4: Harvest supernatant containing virus, aliquot & titer
- Transduce mammalian cells of interest with viral stock
- Select for stably transduced cells using blasticidin
- Assay for protein expressed from CMV promoter



## Virapower Safety Features



- Only 3 HIV genes expressed (gag, pol, rev)
- VSV G replacing HIV envelope protein
- HIV genes split among 4 plasmids with no regions of homology
- No HIV structural genes present in viral progeny
- Gag and Pol gene expression is Rev dependant



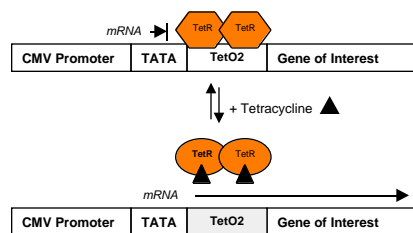
## Gene Expression Issues and Solutions

- **Issues**
  - Expression from the CMV promoter can be down-regulated in certain types of cells over time
- **Solutions**
  - Use non-viral promoters that are expressed in a wide range of host cells can achieve high production levels



## Inducible Mammalian Expression Systems

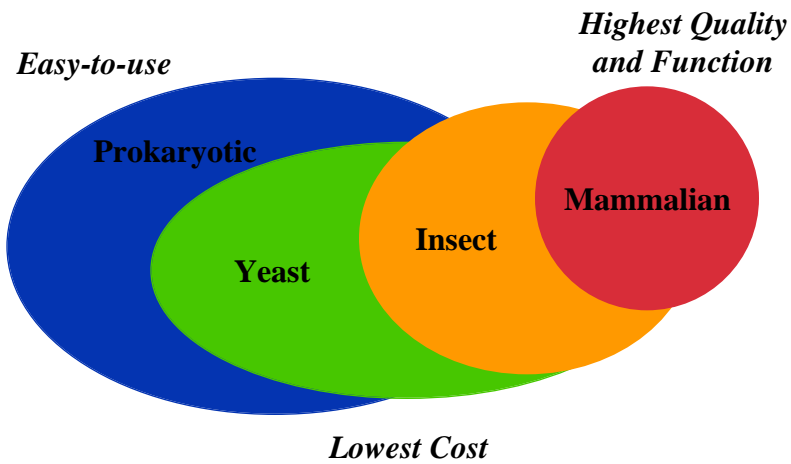
### T-REx™ System



- Transcription is blocked by two TetR homodimers bound to duplicate operator sequences (TetO2).
- Transcription is derepressed when tetracycline binds the TetR dimers causing conformational change and release of the TetO2 sequences.



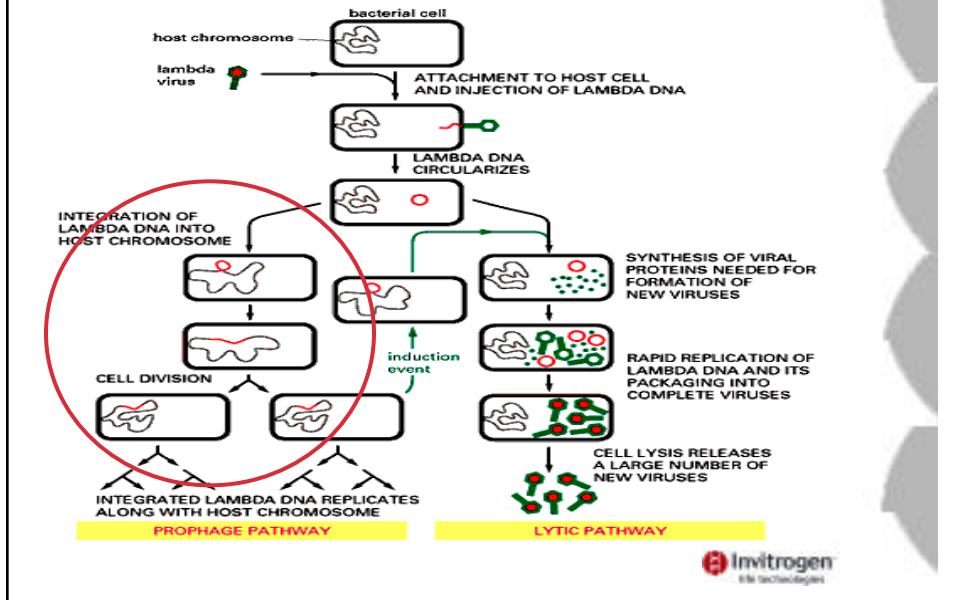
## Gene Expression Systems



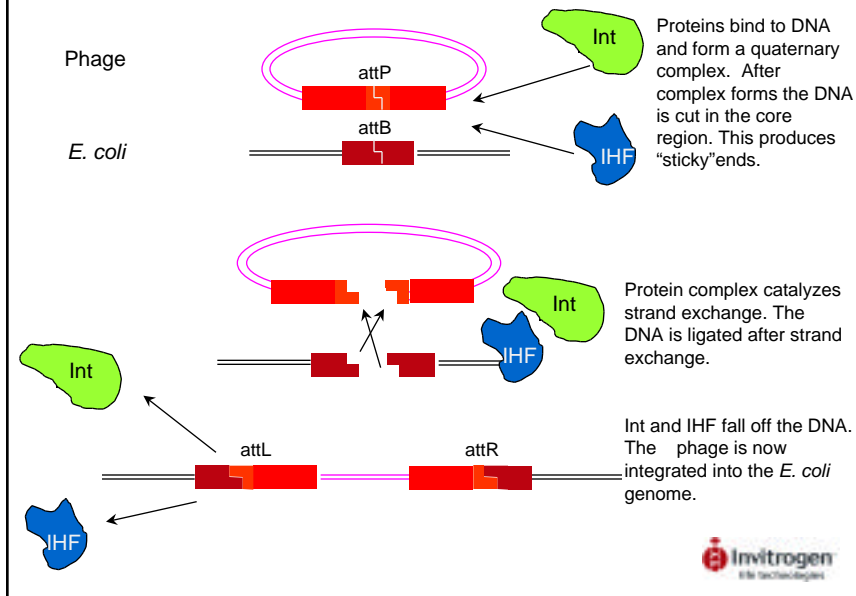
## How Can the Whole Cloning and Expression Process be Accelerated--Gateway Cloning

- One gene, multiple expression systems

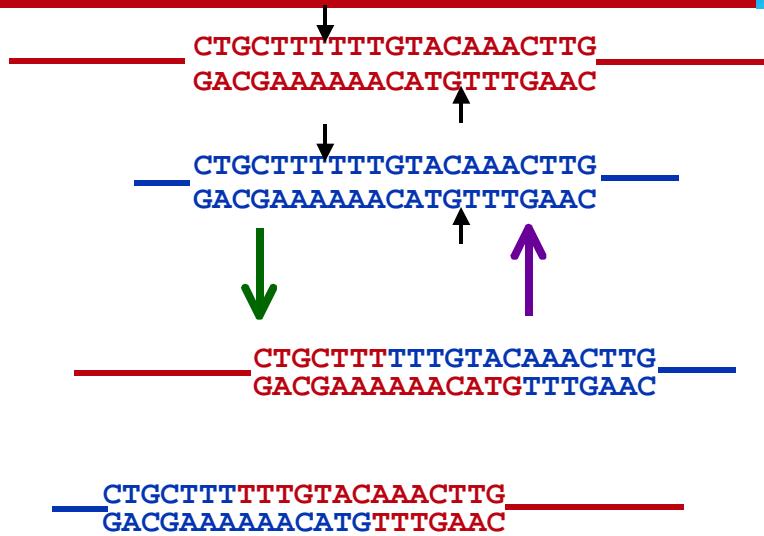
# The Life Cycle of Bacteriophage lambda



# Phage Recombination in *E. coli*



## The Exchange



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## Primer Design for *att B* PCR

- Add the *att B*1 sequence to the 5'-primer
- Add the *att B*2 sequence to the 3'-primer

*att B*1

5' - GGGG ACA AGT TTG TAC **AAA AAA** GCA GGC TNN NNN...

Gene Specific  
Primer Sequence

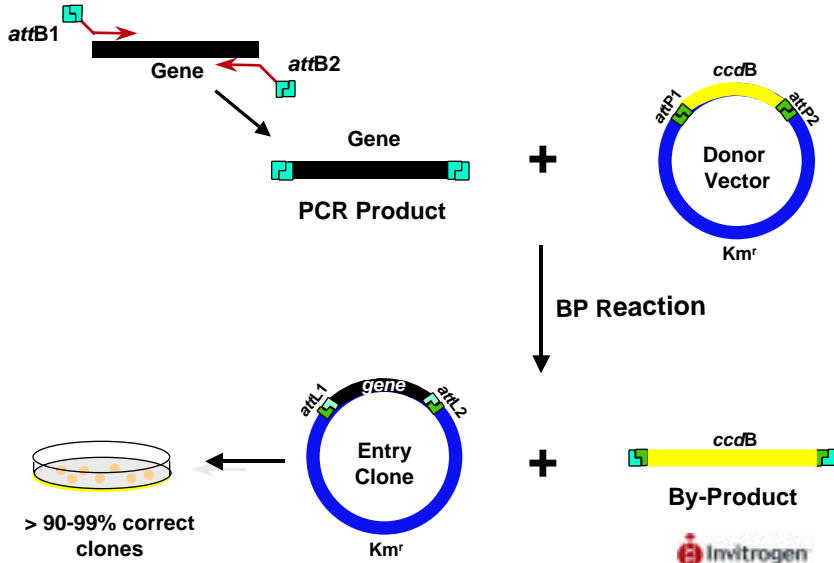
*att B*2

5' - GGGG AC **CAC TTT GTA** CAA GAA AGC TGG **GTN** NNN...

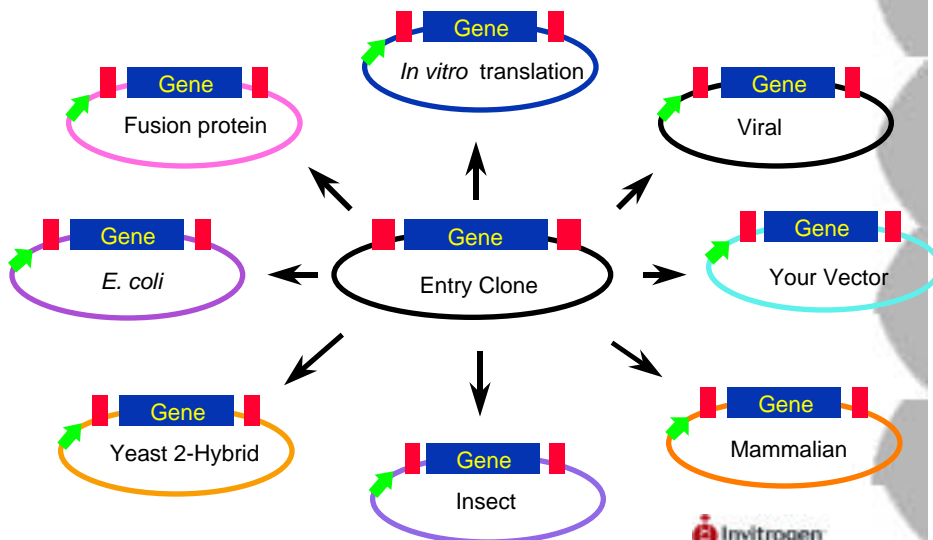
Gene Specific  
Primer Sequence

Invitrogen  
Life Technologies

## Cloning PCR Products



## Subcloning an Entry Clone into Multiple Destination Vectors



## GATEWAY vs Traditional Cloning



### GATEWAY

- Reduces the cloning and subcloning time.
- Once the Entry clone is prepared, subcloning is very fast and efficient.
- The reading frame is considered on the original construction of the Entry clone.
- Once the Entry clone is constructed, the reading frame is maintained on every subcloning and you do not need to consider this in cloning.
- The positive and negative selection used ensures the correct clone is easily found.
- Once the Entry clone is sequence validated, there is no need to sequence any subclones generated.
- The recombination sites are faithfully reproduced in each subcloning.

### Traditional

- Often difficult to find RE sites that maintain the ORF.
- Usually takes 3-4 days to complete.
- Need to sequence the RE site junction to ensure integrity.
- Must plan for every subcloning experiment to ensure RE sites are present and the ORF is maintained.
- If the RE site is present in the ORF then an alternative strategy must be used.