# Gateway®-Technologie: Potenzial und Anwendungen in der molekularen Pflanzenforschung

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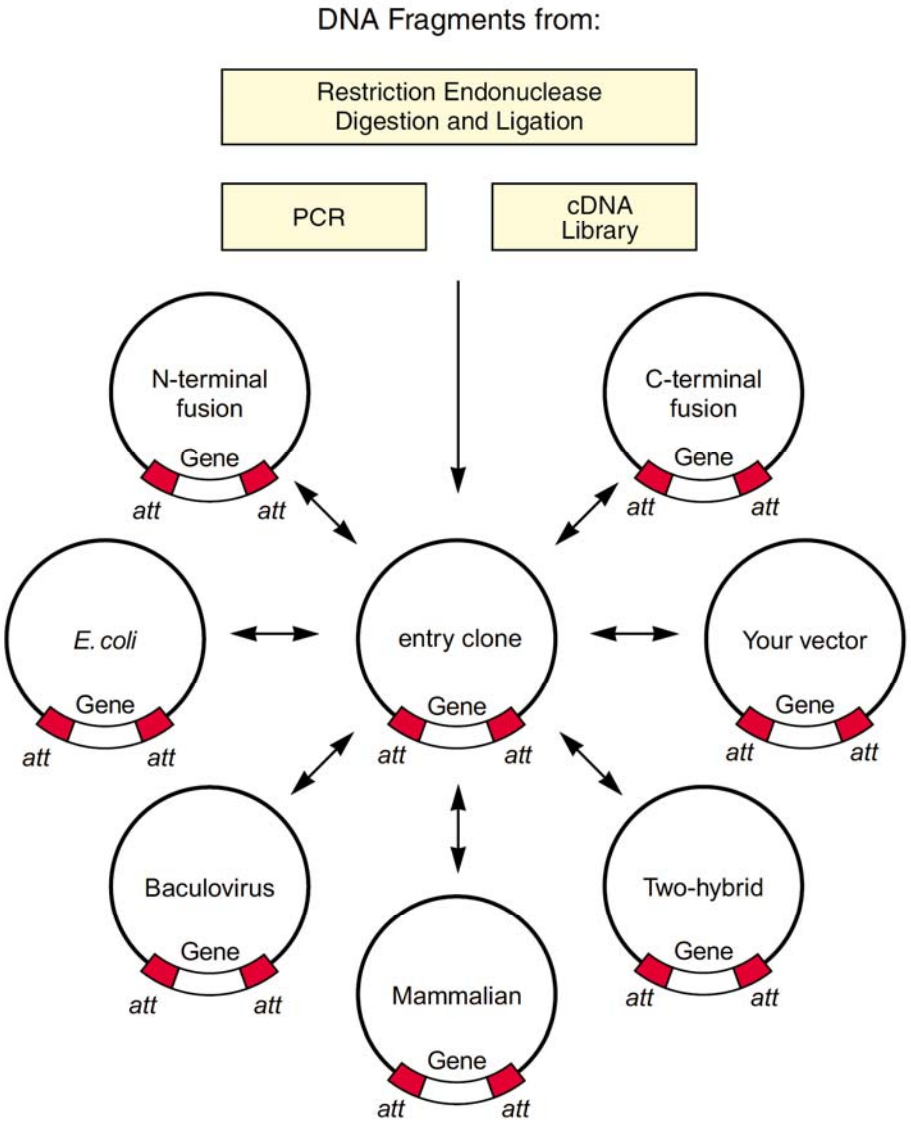
1. Overview and Entry Options

2. Destination Vectors

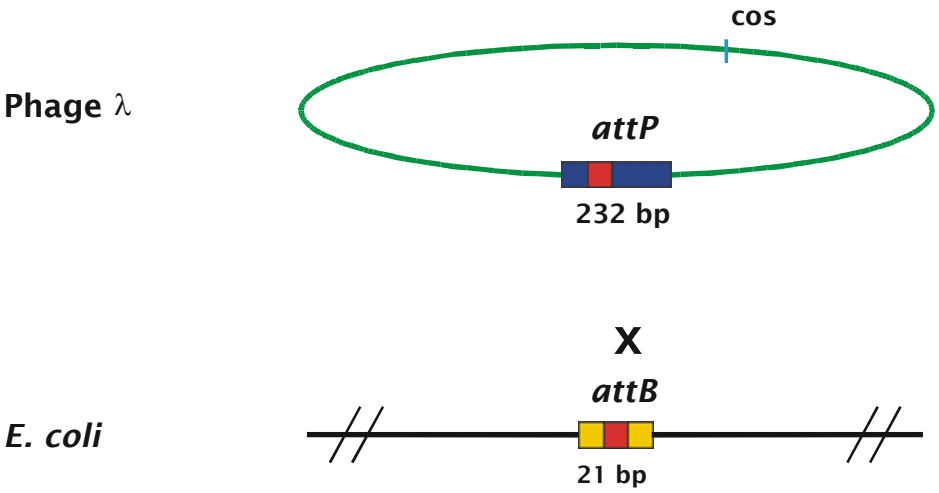
# Key Benefits of the Gateway® Technology

- Efficiently and easily shuttle insert DNA from one expression plasmid to another
- Simplify the cloning workflow and save time
- Create expression clones without using restriction enzymes and ligase
- Utilize ORF clones, a pre-made Gateway® collection
- Simultaneously clone, in a specific order and orientation, up to 4 DNA fragments into one plasmid

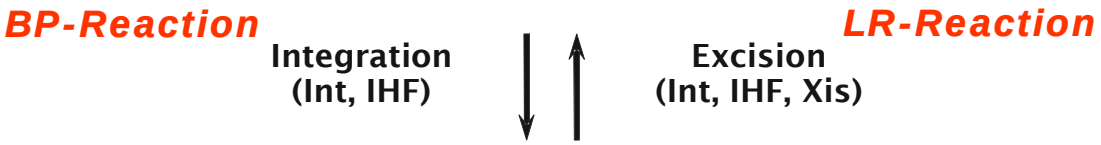
# Gateway® Technology : Overview



# Phage lambda recombination in *E. coli*




**The Gateway® System relies on five sets of specific and non cross-reacting *att* sequences**




# Characteristics of the Modified *att* Sites

| Site | Length | Found in… |
|---|---|---|
| *att*B | 25 bp | Expression vector |
| | | Expression clone |
| *att*P | 200 bp | Donor vector |
| *att*L | 100 bp | Entry vector |
| | | Entry clone |
| *att*R | 125 bp | Destination vector |

**Specificity:**

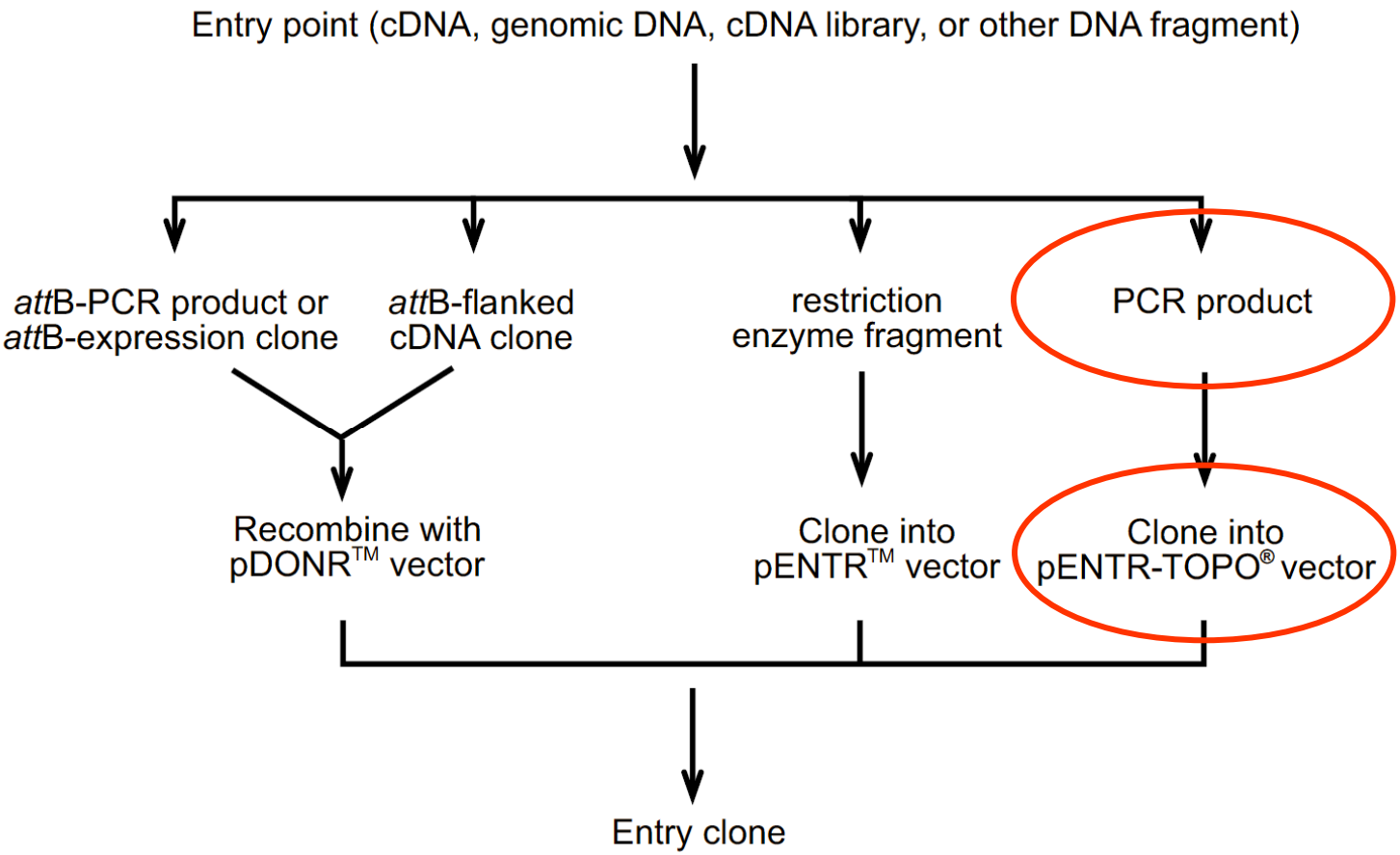
- *att*B1 sites react only with *att*P1 sites
- *att*B2 sites react only with *att*P2 sites
- *att*L1 sites react only with *att*R1 sites
- *att*L2 sites react only with *att*R2 sites

# Options to Create Entry Clones

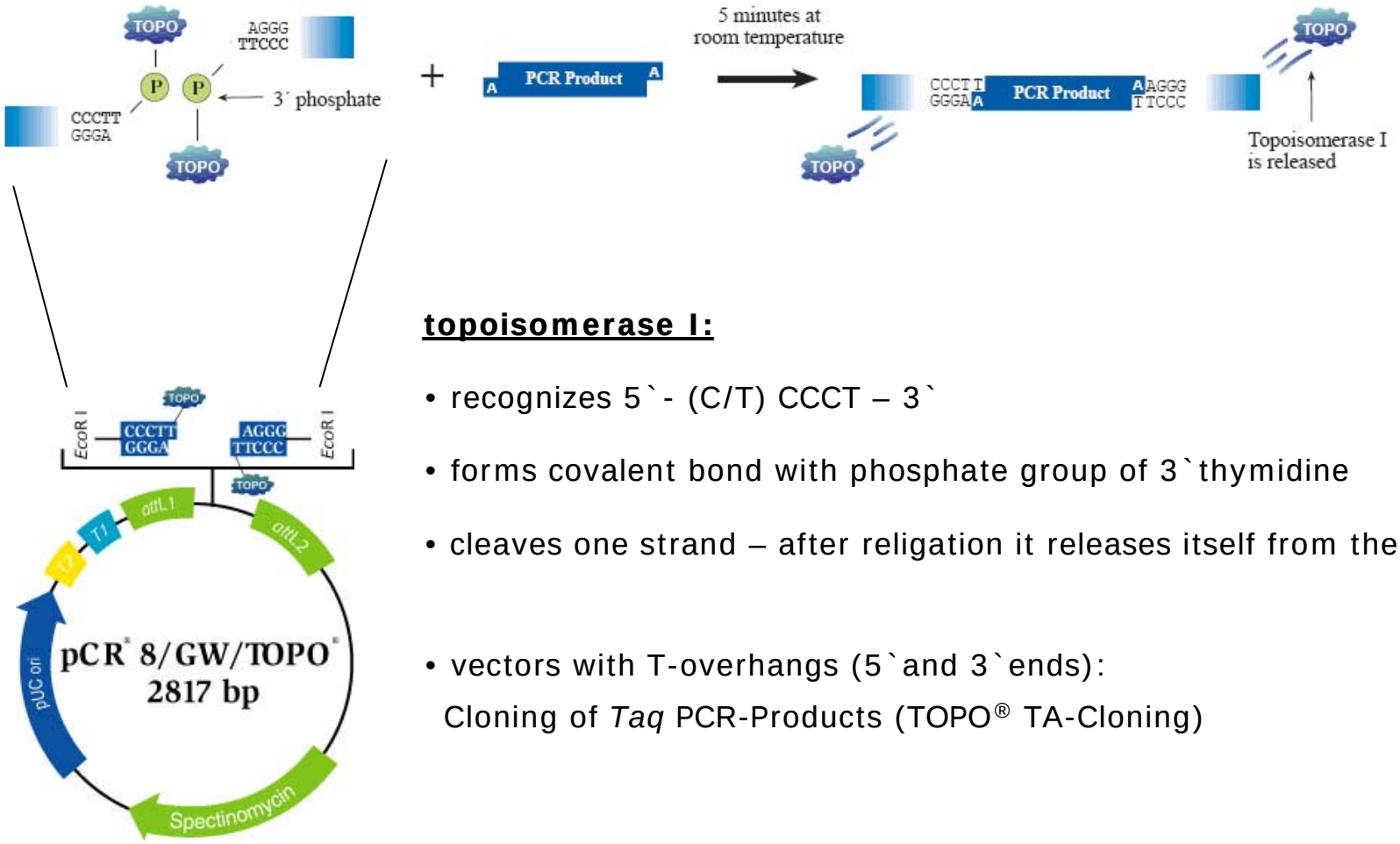
| Entry Vector | Kozak | Shine-Dalgarno |
|---|---|---|
| pENTR/D-TOPO® | • | |
| pENTR/SD/D-TOPO® | • | • |
| pENTR™1A | • | • |
| pENTR™2B | • | |
| pENTR™3C | • | • |
| pENTR™4 | • | |
| pENTR™11 | • | • |

| Vector | M13 Sequencing Sites | Selection Marker |
|---|---|---|
| pDONR™201 | No | Kanamycin |
| pDONR™221 | Yes | Kanamycin |
| pDONR™/Zeo | Yes | Zeocin™ |

# Options to Create Entry Clones I



# TOPO® - Cloning: TOPO®TA

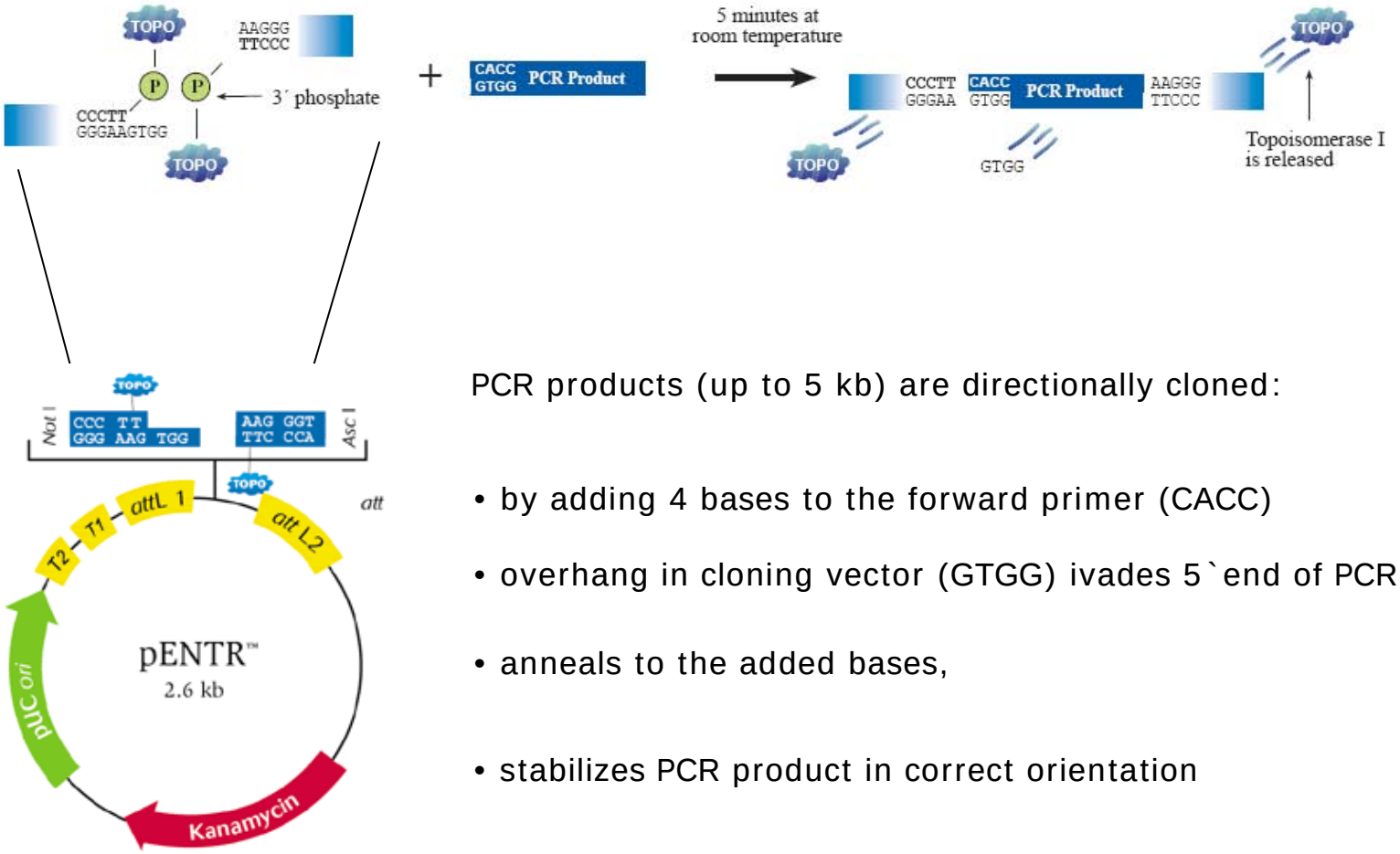



**topoisomerase I:**

- recognizes 5`- (C/T) CCCT – 3`
- forms covalent bond with phosphate group of 3`thymidine
- cleaves one strand – after religation it releases itself from the DNA

- vectors with T-overhangs (5`and 3`ends):
  Cloning of *Taq* PCR-Products (TOPO® TA-Cloning)

# PCR-Directional TOPO® Cloning



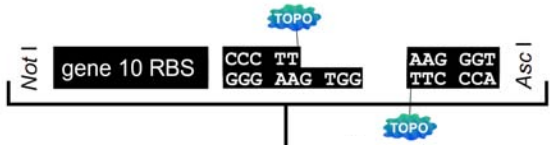

PCR products (up to 5 kb) are directionally cloned:

- by adding 4 bases to the forward primer (CACC)
- overhang in cloning vector (GTGG) ivades 5`end of PCR-product,
- anneals to the added bases,
- stabilizes PCR product in correct orientation

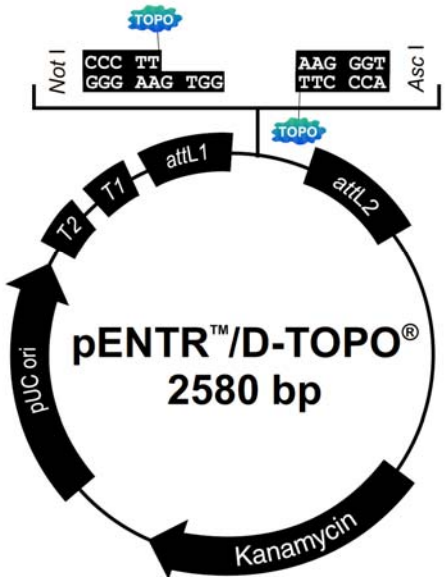
# PCR-Directional TOPO® Cloning

**A**

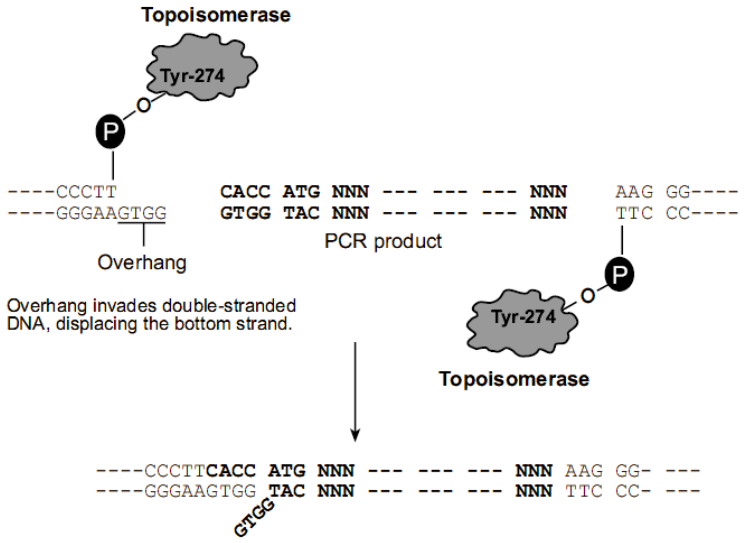
## pENTR/SD/D-TOPO



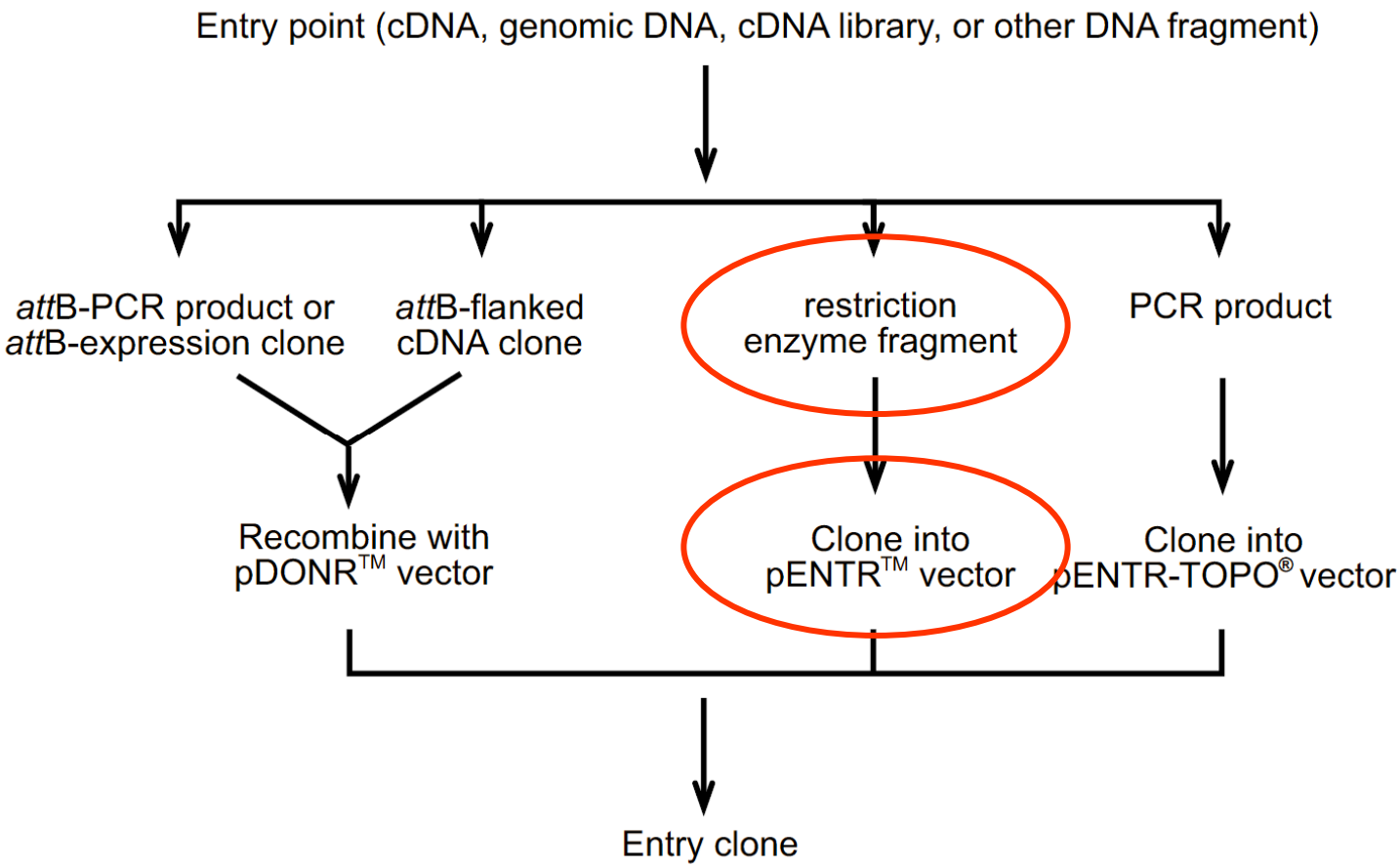

## pENTR/D/D-TOPO



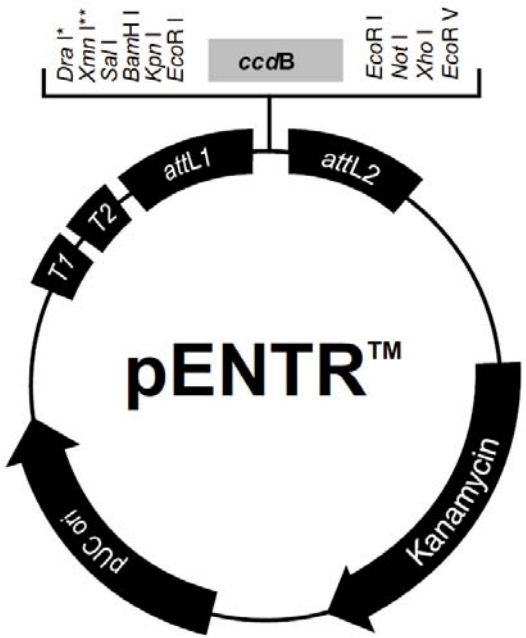

**B**



# Options to Create Entry Clones II



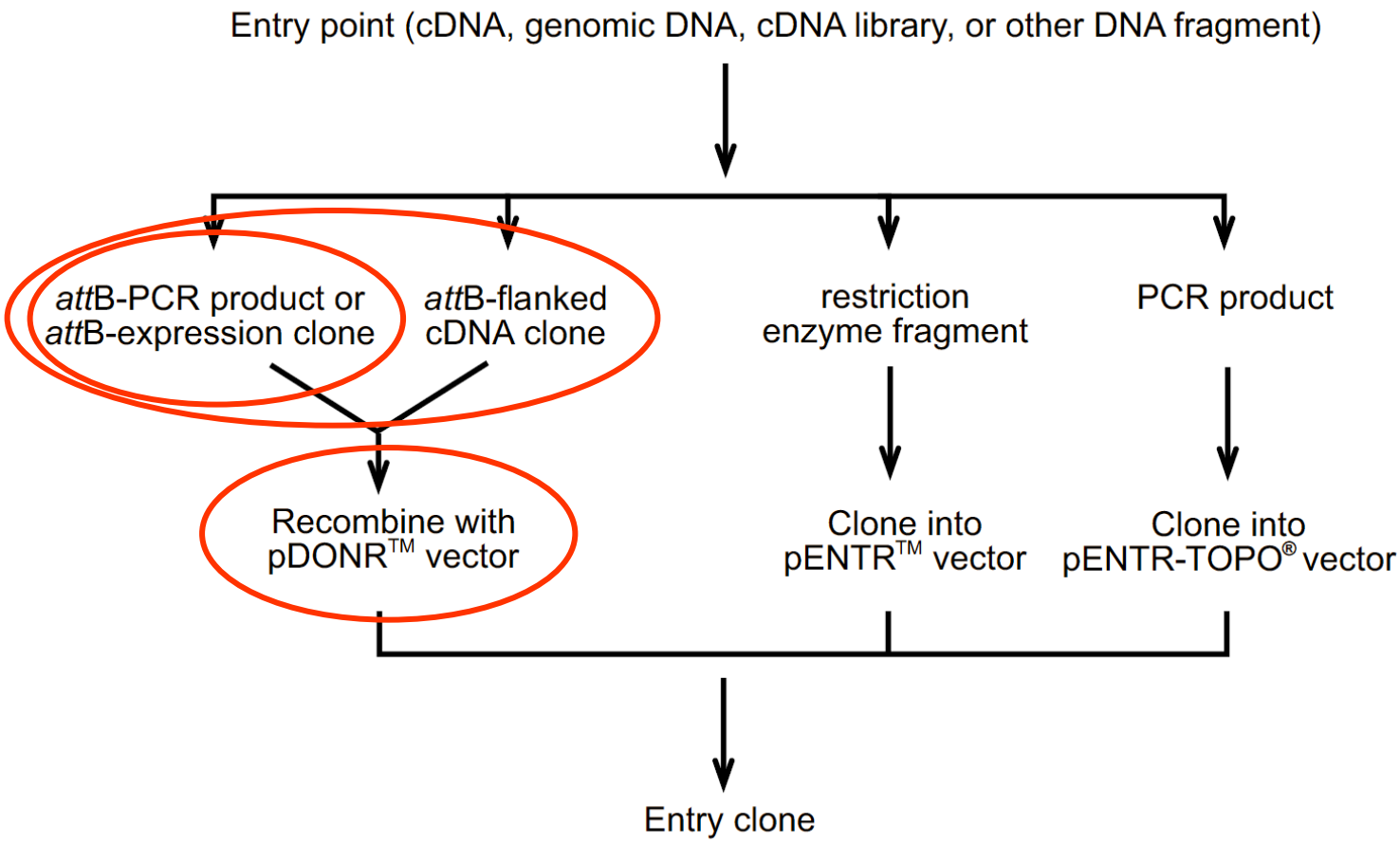
# Restriction / Ligase Cloning



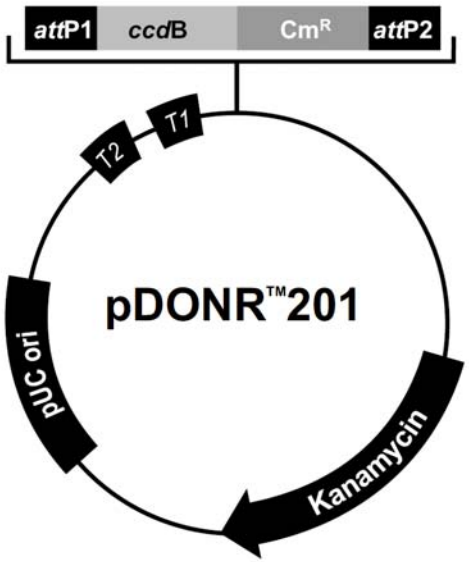

- Use when there are convenient sites to cut insert out of another plasmid
- Must cut out *ccdB* gene by using one of four RE sites flanking the ccdB
- Reading frame of insert must be considered, as well as downstream expression elements
- Various reading frames of pENTR vectors are available

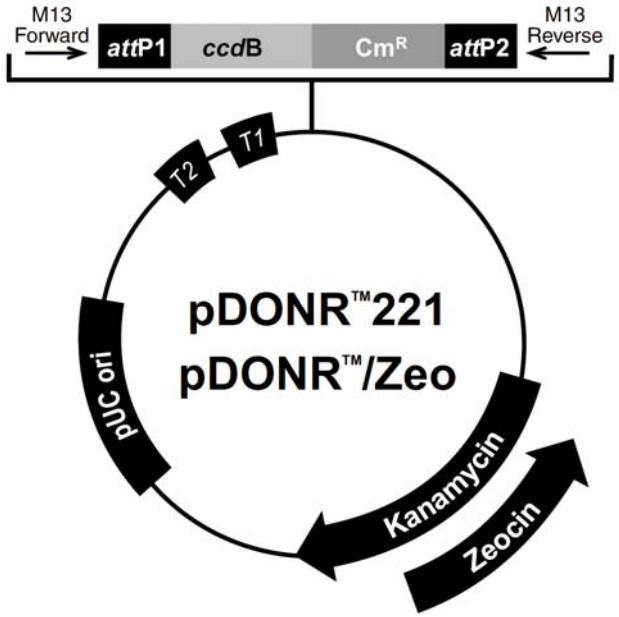
| | |
|---|---|
| **pENTR™ 1A** | **Vector in reading frame 0** |
| **pENTR™ 2B** | **Vector in reading frame +1** |
| **pENTR™ 3C** | **Vector in reading frame +2** |
| **pENTR™ 4** | **Vector in reading frame 0; modified polylinker from 1A** |
| **pENTR™ 11** | **Contains both an *E. coli* (SD) and eukoryotic (Kozak) ribosome binding site** |

# Options to Create Entry Clones III



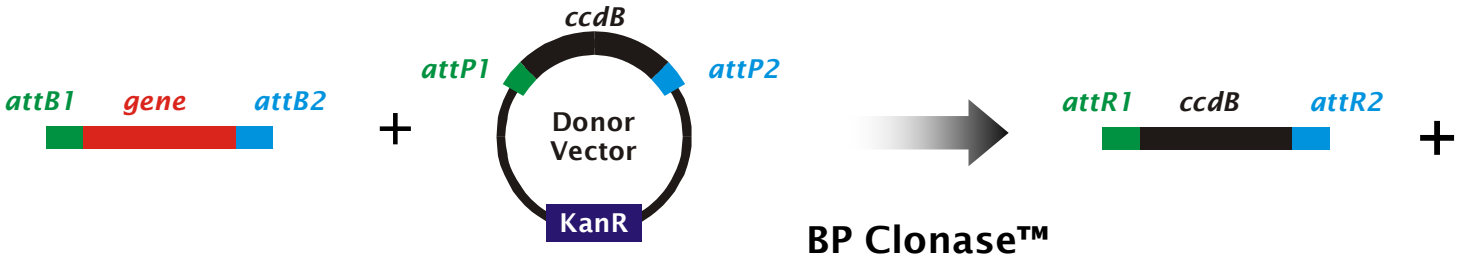
# Map and Features of pDONR 201, pDONR221 and pDONR/Zeo

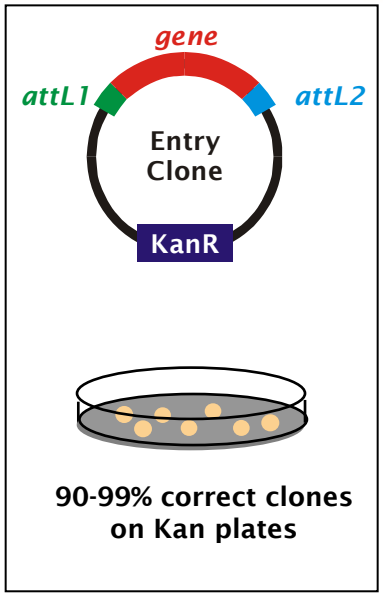



- *rrn* BT2 transcription termination sequence
- *rrn* BT1 transcription termination sequence
- attP1 site
- *ccdB* gene
- chloramphenicol resistance gene
- attP2 site
- kanamycin resistance gene } pDONR201 and pDONR221
- pUC origin
- T7 promotor / priming site } pDONR221 and pDONR/Zeo
- M13 reverse priming site } pDONR221 and pDONR/Zeo
- Zeocin resistance gene } pDONR/Zeo

# BP Cloning – The Reaction




# BP Cloning - Primer Design for PCR

## To be considered when designing PCR Primers:

- Sequences to facilitate Gateway cloning

- Sequences required for efficient expression of the native protein (i.e. Shine-Dalgarno or Kozak consensus)

- Whether or not you wish your PCR product to be fused in frame with an N- or C-terminal fusion tag

# BP Cloning - Primer Design for PCR

- GGGG and the *att*B1 sequence must be added to the 5'-primer (sense)
- GGGG and the *att*B2 sequence must be added to the 3'-primer (antisense)

*Forward Primer:*

*att*B1

5' – GGGGACAAGTTTGTACAAAAAAGCAGGCTNNN...

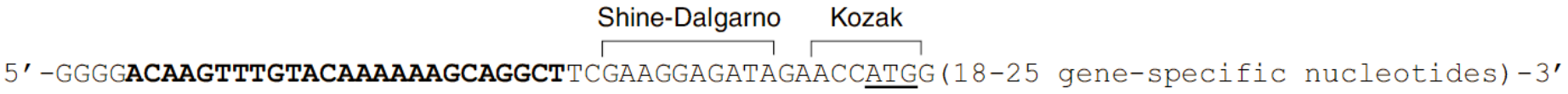
*Reverse Primer:*

*att*B2

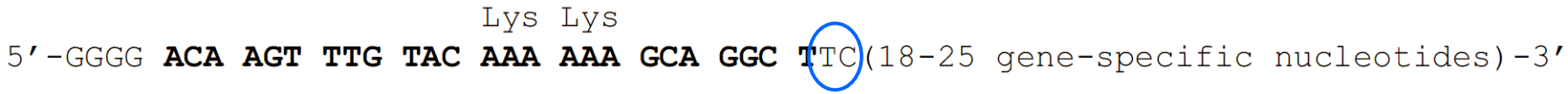
5' – GGGGACCACTTTGTACAAGAAAGCTGGGTNNN...

# Designing *attB* Forward Primers

## 1) Forward Primer Design for Native Expression




## 2) Forward Primer Design for N-terminal Fusions



# Designing *attB* Reverse Primers

**1)** Reverse Primer Design with no C-terminal fusion tag

5′-GGGG**ACCACTTTGTACAAGAAAGCTGGGT**CCTA(18-25 gene-specific nucleotides)-3′

***attB2***

**2)** Reverse Primer Design for C-terminal fusion tag

5′-GGGG-AC-CAC-**TTT-GTA**-CAA-GAA-AGC-TGG-GTN--(template-specific sequence)-3′

*att*B2

# Gateway® BP Clonase Reaction

| | | |
|---|---|---|
| *attB*-PCR Product | (30 - 300 ng) | 1 – 10 µl |
| Donor vector | (150 ng/µl) | 2 µl |
| 5x BP Clonase Reaction Buffer | | 4 µl |
| TE, pH 8.0 | | 16 µl |

→ + 4 µl BP Clonase enzyme mix

→ 25°C, over night

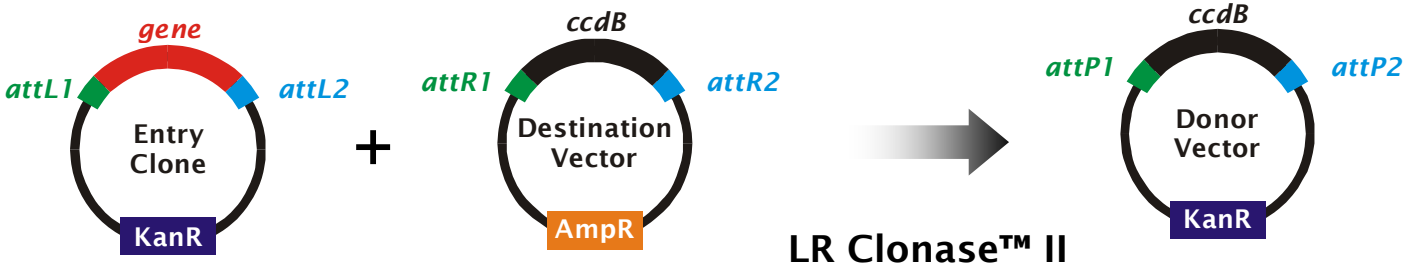
→ + 2 µl Proteinase K

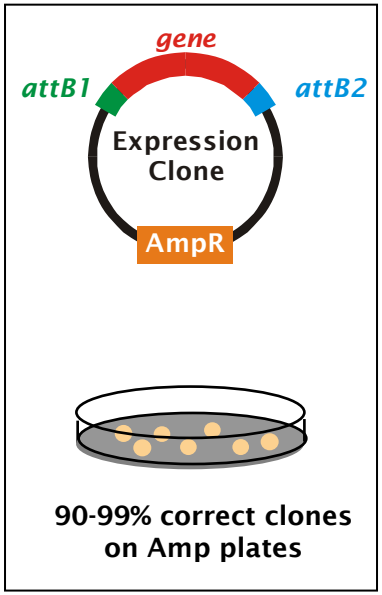
→ 37°C, 10 min

**BP Clonase Mix:** Reaction Buffer + Clonase Enzyme Mix

**BP Clonase II Mix:** Clonase Mix includes Reaction Buffer, (less expensive)

# Gateway® LR-Recombination Reaction



# *E. coli* strains for transformation of Gateway constructs

| | |
|---|---|
| **OmniMAX 2-T1® :** | Selection of (positive) transformants, „general“ cloning strain |
| **DH5α:** | Selection of (positive) transformants, „general“ cloning strain |
| ***ccdB*-survival :** | To maintain Gateway – constructs without insert |
| **DB3.1:** | To maintain Gateway – constructs without insert |

# Gateway®-Technology: Summary

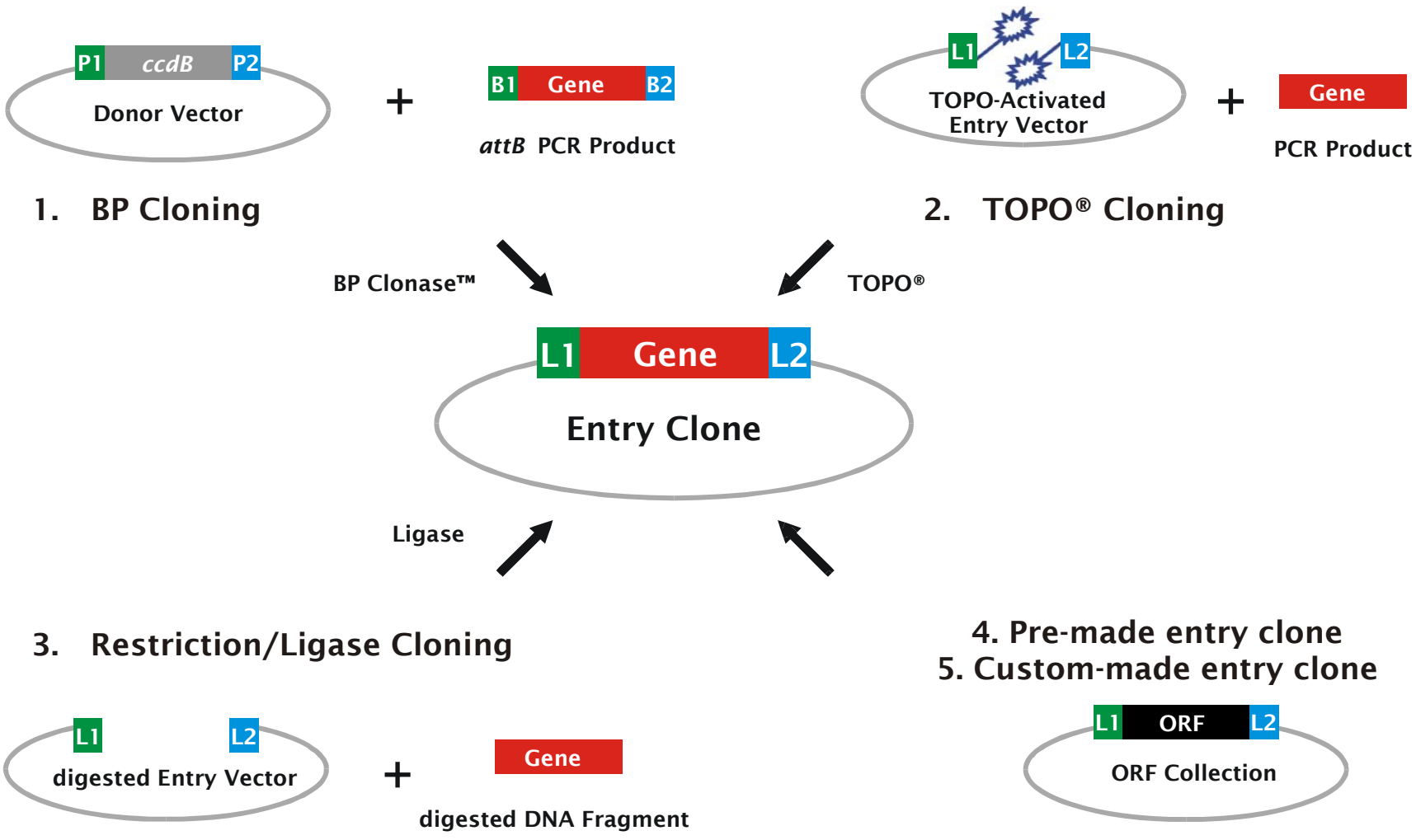
1.


- **LR Reaction:** Facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone (see diagram below). This reaction is catalyzed by LR Clonase™ enzyme mix.

2.


| | Pathway | Reaction | Catalyzed by… |
|---|---|---|---|
| 1. | Lysogenic | *att*B x *att*P → *att*L x *att*R | BP Clonase™ (Int, IHF) |
| 2. | Lytic | *att*L x *att*R → *att*B x *att*P | LR Clonase™ (Int, Xis, IHF) |

# Different ways to generate the entry clone



1. Overview and Entry Options

2. Destination Vectors

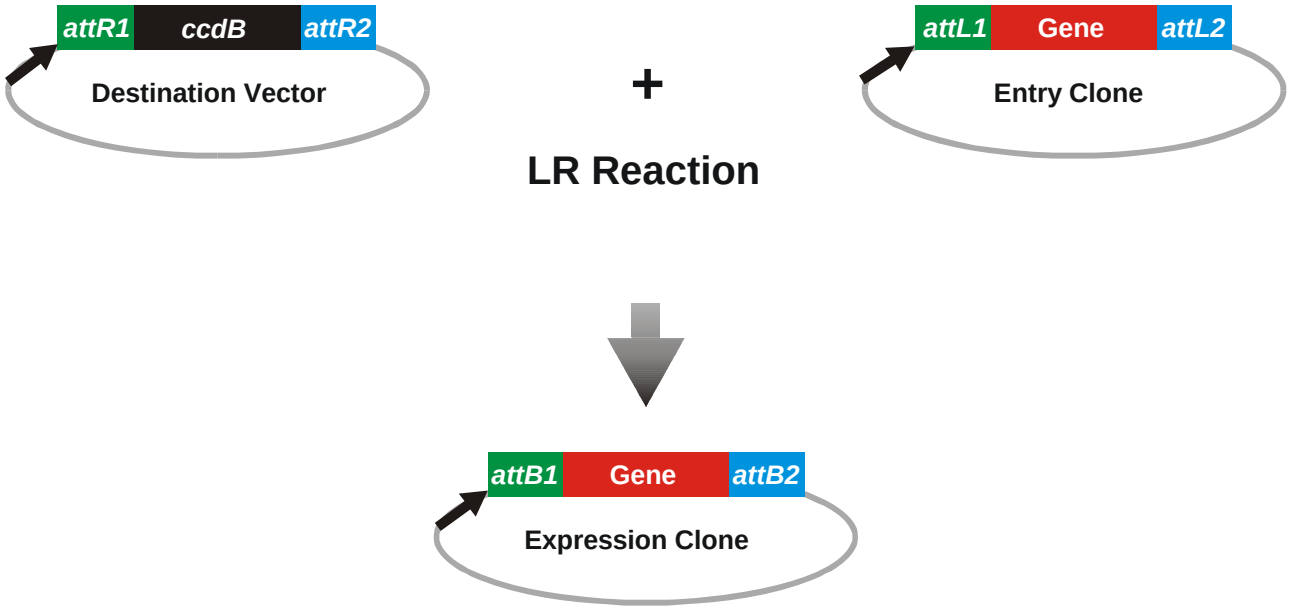



Fig. ©Invitrogen

## destination vectors:

- have *attR* sites
- allow recombination with entry clones

# Number of applications by Invitrogen

Table 2—Gateway® expression vector options.

| Application | Gateway® Destination vector family |
|---|---|
| Protein array | Expressway™ Plus Expression system |
| Antibody or antigen production | Champion™ pET Expression systems |
| Protein expression in *E. coli* | pDEST™14, 15, 17, and 24<br>pET160 and pET161 DEST™ vectors |
| Protein expression in yeast | pYES2-DEST™52 |
| Protein expression in insect cells | BaculoDirect™ C-term Expression Kit |
| Protein expression in mammalian cells (constitutive expression) | pcDNA® mammalian expression vector family |
| Protein expression in mammalian cells (regulated expression) | pT-REx-DEST30 and pT-REx-DEST31 vectors |
| Protein expression in mammalian cells (viral delivery) | ViraPower™ Lentiviral Expression Systems |
| Protein-protein interaction studies | ProQuest™ Two-Hybrid System using Gateway® technology |
| Localization | VividColors™ pcDNA GFP Destination vector family |
| RNAi | BLOCK-iT™ vector family |
| Reporter assay | GeneBLAzer™ pcDNA vector family |

# Destination vectors for protein expression

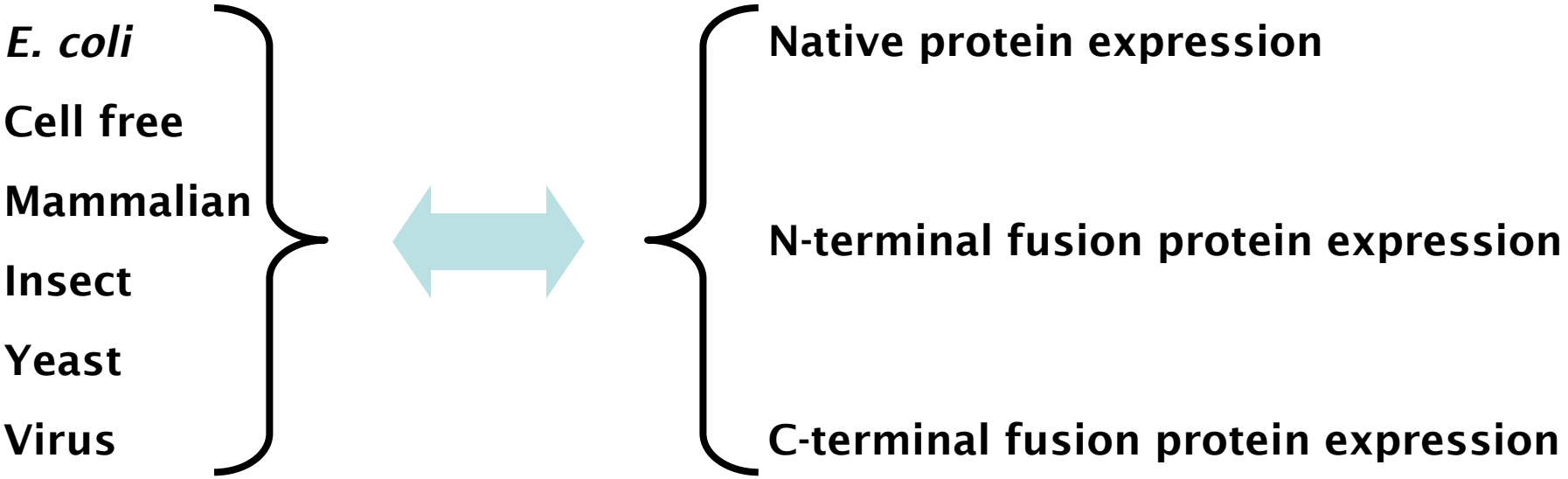
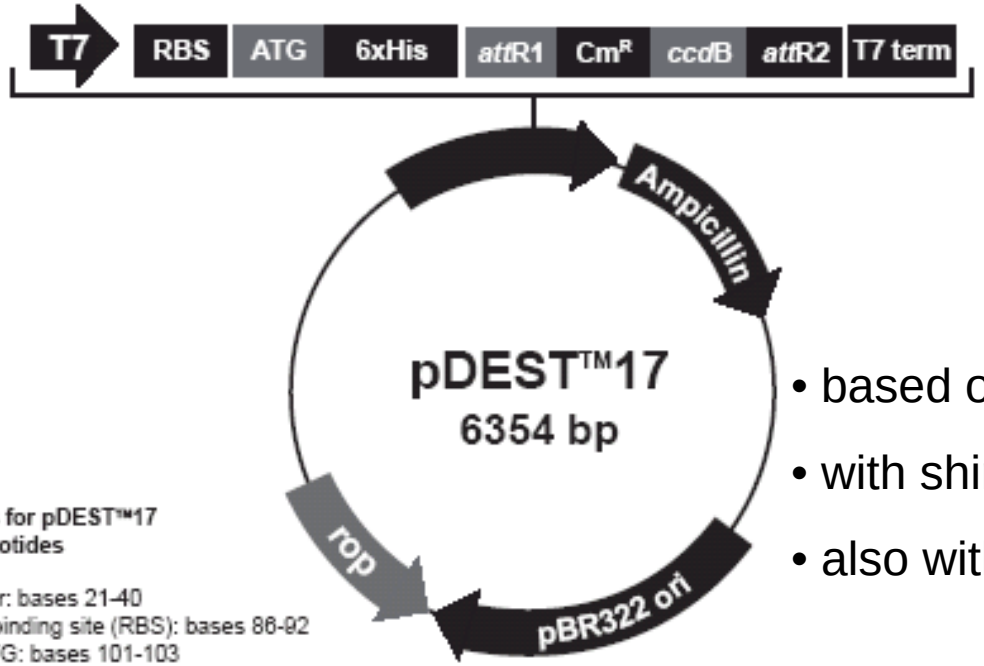



Fig. ©Invitrogen

# *E. coli:* pDEST17




- based on pET vectors
- with shine dalgarno sequence
- also with C-term Tag or GST-Tags

**Comments for pDEST™17**
**6354 nucleotides**

T7 promoter: bases 21-40
Ribosome binding site (RBS): bases 86-92
Initiation ATG: bases 101-103
6xHis tag: bases 113-130
*attR1*: bases 140-264
Chloramphenicol resistance gene ($Cm^R$): bases 373-1032
*ccdB* gene: bases 1374-1679
*attR2*: bases 1720-1844
T7 transcription termination region: bases 1855-1983
*bla* promoter: bases 2471-2569
Ampicillin (*bla*) resistance gene: bases 2570-3430
pBR322 origin: bases 3575-4248
*ROP* ORF: bases 4619-4810 (C)

C=complementary strand

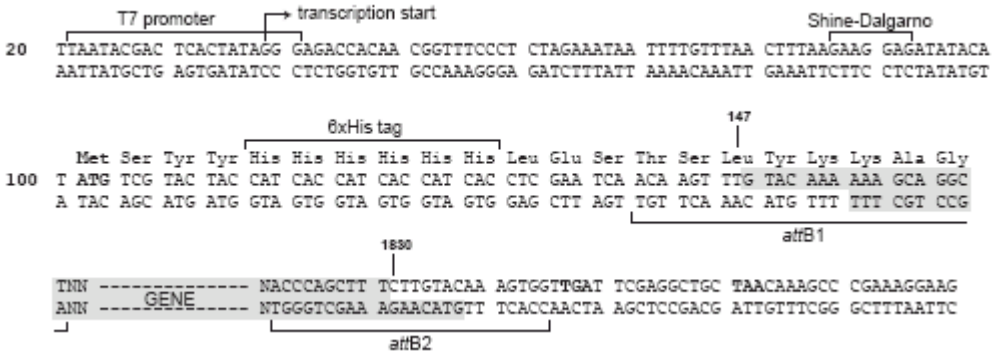



Figure & Sequence
©Invitrogen

# overview about other destination vectors

- *in vitro* Transcription/ Translation
- protein expression in other organisms (e.g. yeast, mammalian, insect cells)
- Two-Hybrid System in Yeast
- RNAi

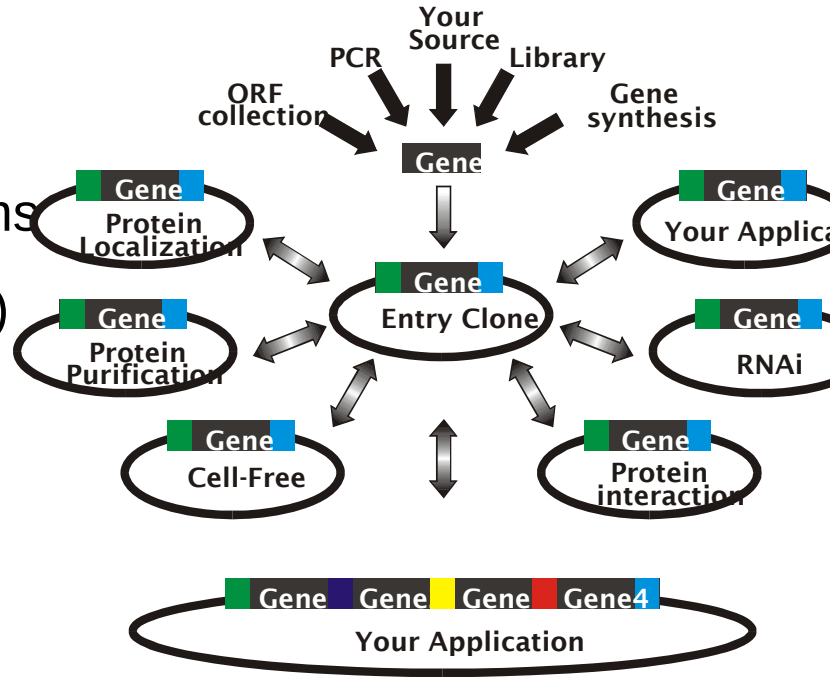


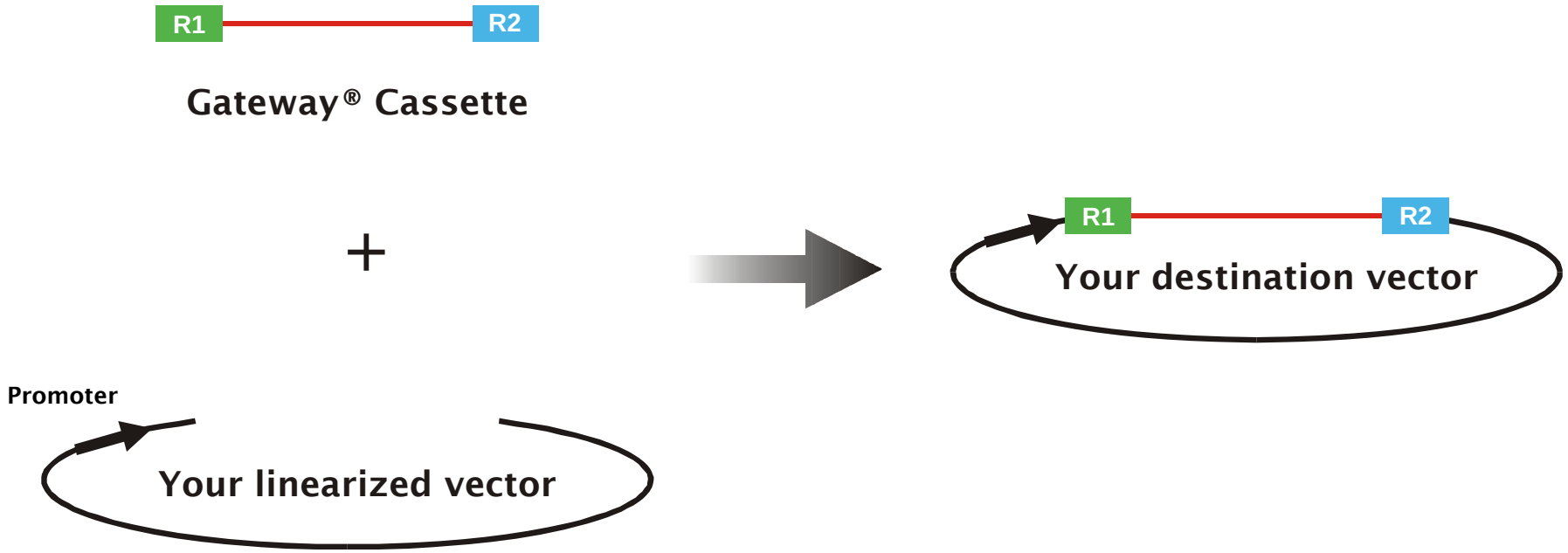

Fig. ©Invitrogen

➔Creation of your own Gateway-vector:

conversion cassette

→ in plants: based on pCAMBIA

# Gateway® Conversion Kit



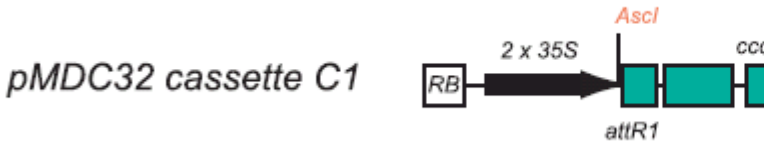

**Conversion cassettes can be used with any vector or system, even the proprietary ones**

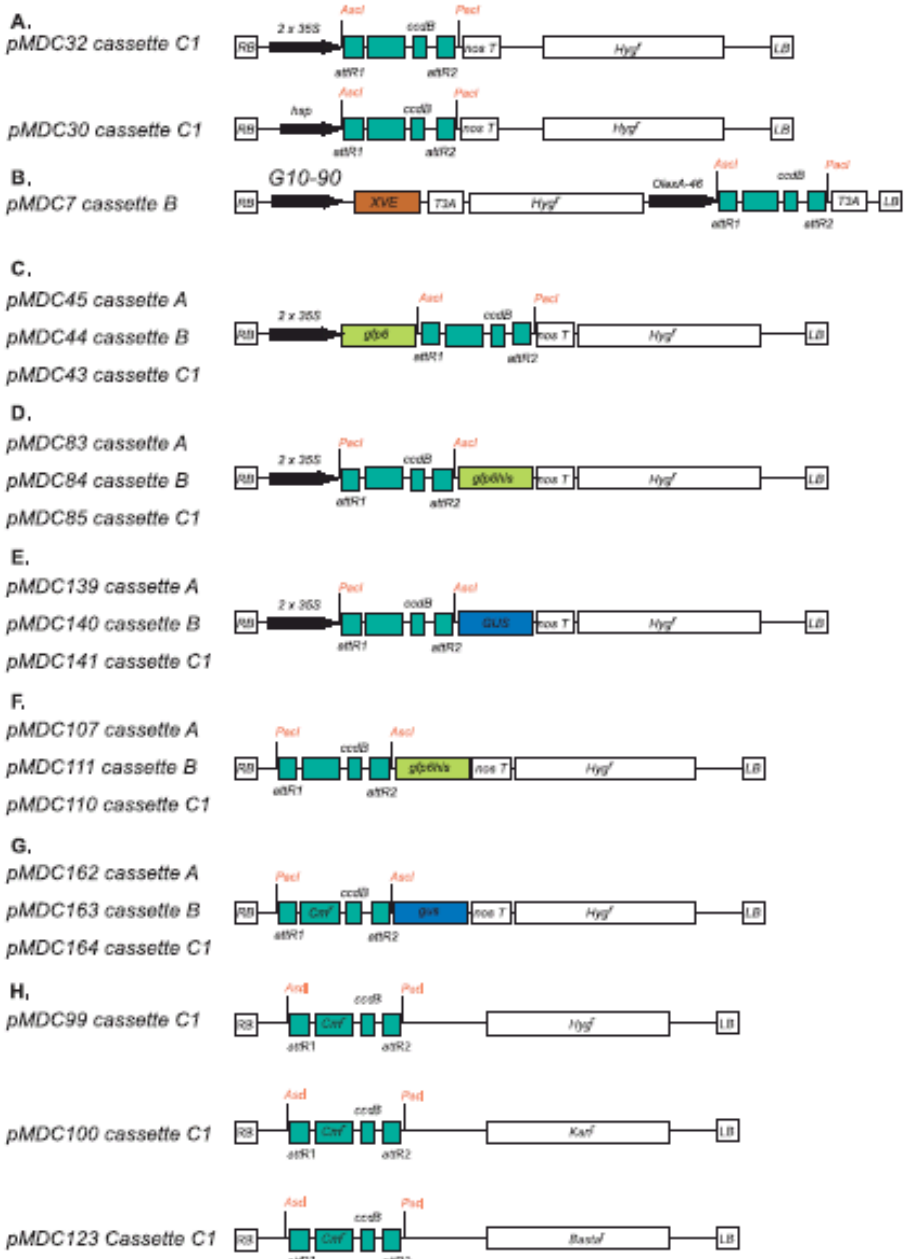
# examples for created plant transformation vectors

- pMDC Gateway Vector Series

  (Curtis & Grossniklaus (*2003) Plant Phy*

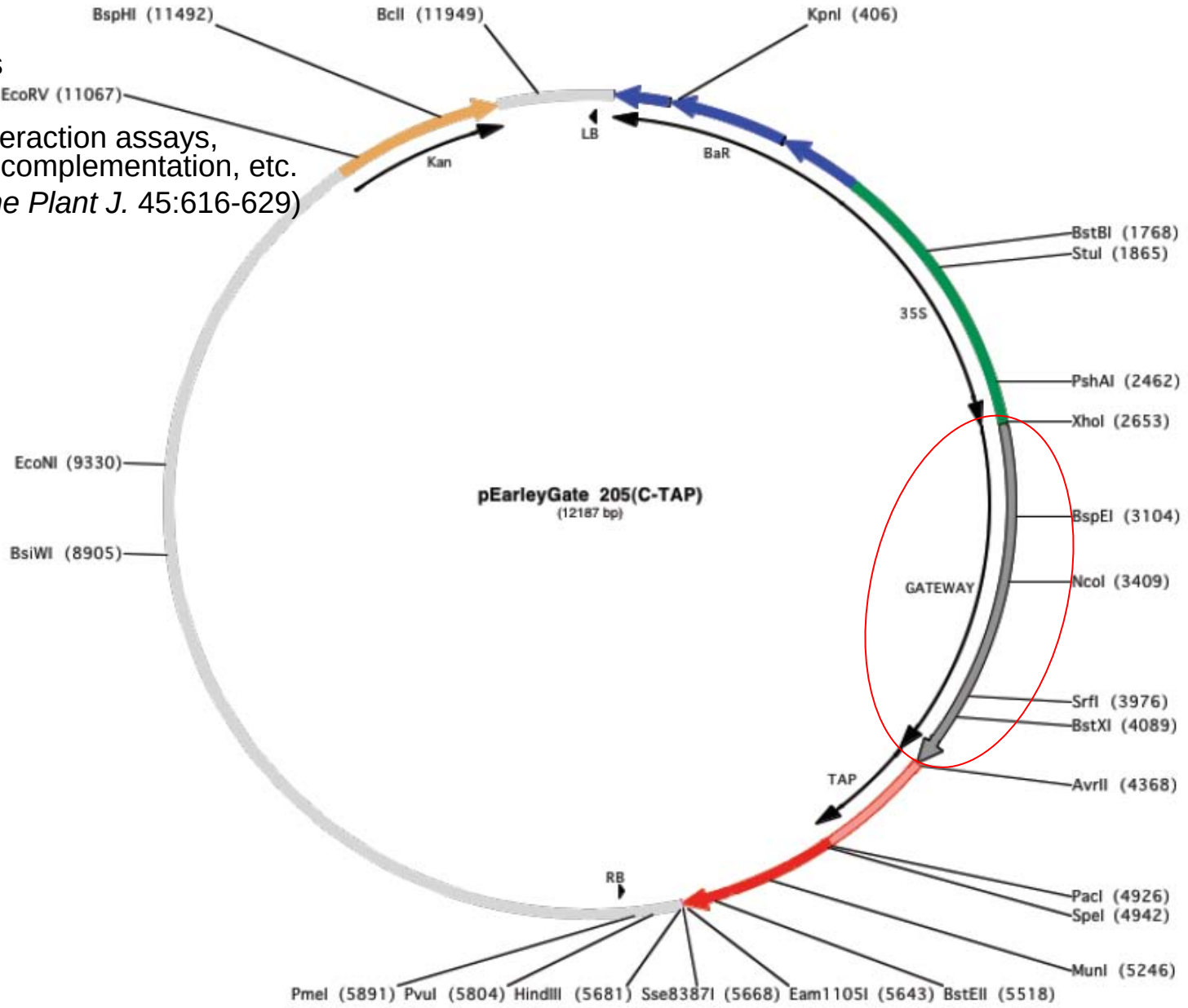
  → Complementation



# pEarleyGate Vector Series: pEarleyGate 205

pEarley Vector Series
by Pikaard laboratory
→ Purification and Interaction assays, Fluorescent Imaging, complementation, etc.
(Earley *et al*. (2006) *The Plant J.* 45:616-629)

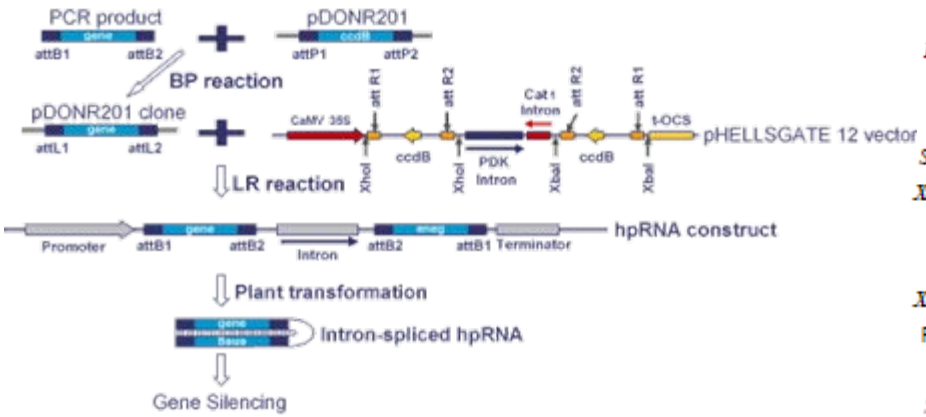


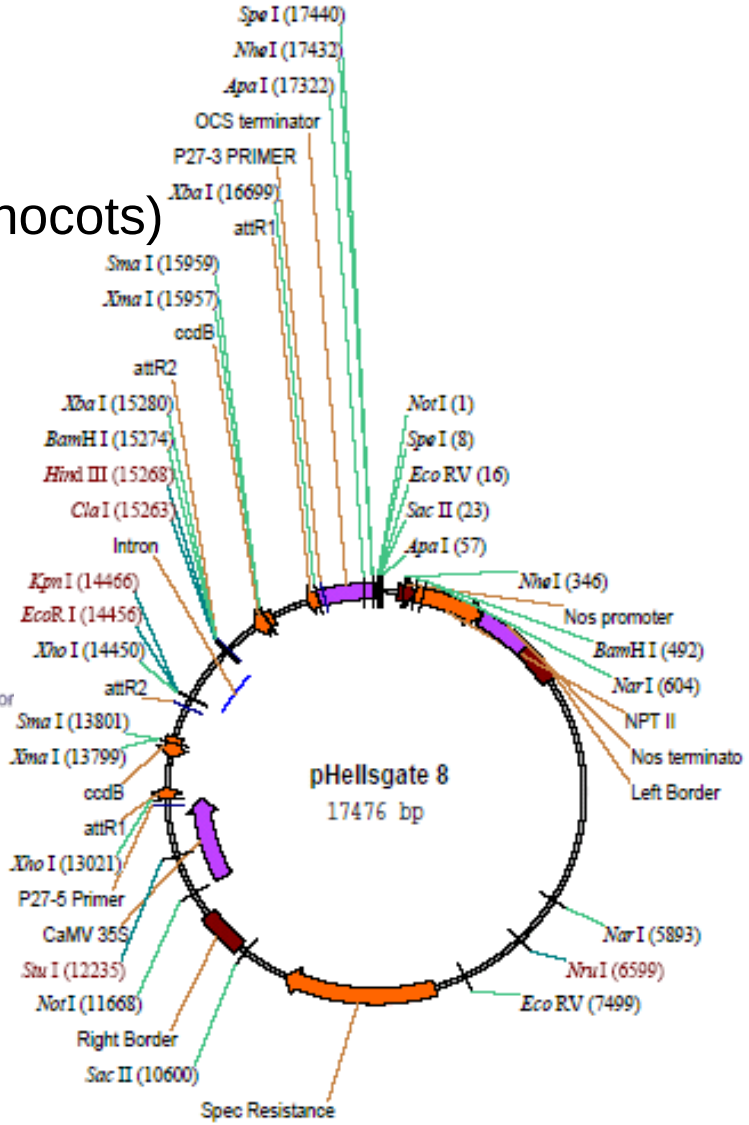
# pEarleyGate Vector Series: overview

| Plasmid name | Description |
|---|---|
| pEarleyGate 100 | 35S-Gateway-OCS 3' |
| pEarleyGate 101 | 35S-Gateway-YFP-HA tag-OCS 3' |
| pEarleyGate 102 | 35S-Gateway-CFP-HA tag-OCS 3' |
| pEarleyGate 103 | 35S-Gateway-GFP-His tag- OCS 3' |
| pEarleyGate 104 | 35S-YFP-Gateway-OCS 3' |
| pEarleyGate 201 | 35S-HA tag-Gateway-OCS 3' |
| pEarleyGate 202 | 35S-FLAG tag-Gateway-OCS 3' |
| pEarleyGate 203 | 35S-Myc tag-Gateway-OCS 3' |
| pEarleyGate 204 | 35S-AcV5 tag-Gateway-OCS 3' |
| pEarleyGate 205 | 35S-Gateway-TAP tag-OCS 3‘ |
| pEarleyGate 301 | no promoter-Gateway-HA tag-OCS 3' |
| pEarleyGate 302 | no promoter-Gateway-FLAG tag-OCS 3' |
| pEarleyGate 303 | no promoter-Gateway-Myc tag-OCS 3' |
| pEarleyGate 304 | no promoter-Gateway-AcV5 tag-OCS 3' |

# RNAi *in planta:* pHellsgate series

- hairpinRNAi vectors for plants
- pHellsgate (Dicots) vs. pStargate (Monocots)

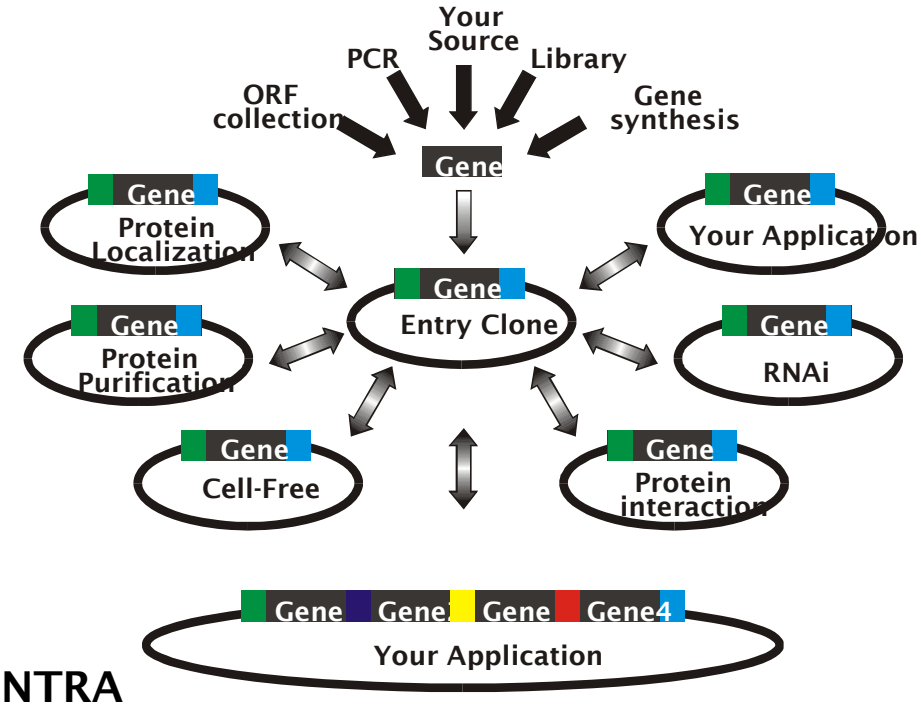


# conclusion of The Gateway® Cloning System

**PRO**

- Directional cloning
- Maintains reading frame
- No restriction enzymes
- No ligation
- 1 hour, room-temperature reaction with >99% efficiency
- No re-sequencing
- Compatible with automation
- Reversible reactions




**CONTRA**

- Primer
- dependance on Invitrogen (LR/BP reaction mix)
- antibiotics resistance

Thank you for

your attention!

## Weiterführende Internet-Seiten der Firma Invitrogen:

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning.html

http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf/Brochures.Par.38170.File.dat/B-074573-Gateway.pdf

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Gateway-Cloning/GatewayC-Misc/Online-Seminars.html