National Cancer Institute

Gateway Cloning for Mouse 101

Dominic Esposito, Ph.D.

Principal Scientist
Group Leader, Clone Optimization Group
Protein Expression Laboratory
Advanced Technology Program
SAIC-Frederick, Inc.
National Cancer Institute at Frederick
Frederick, MD, USA

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health

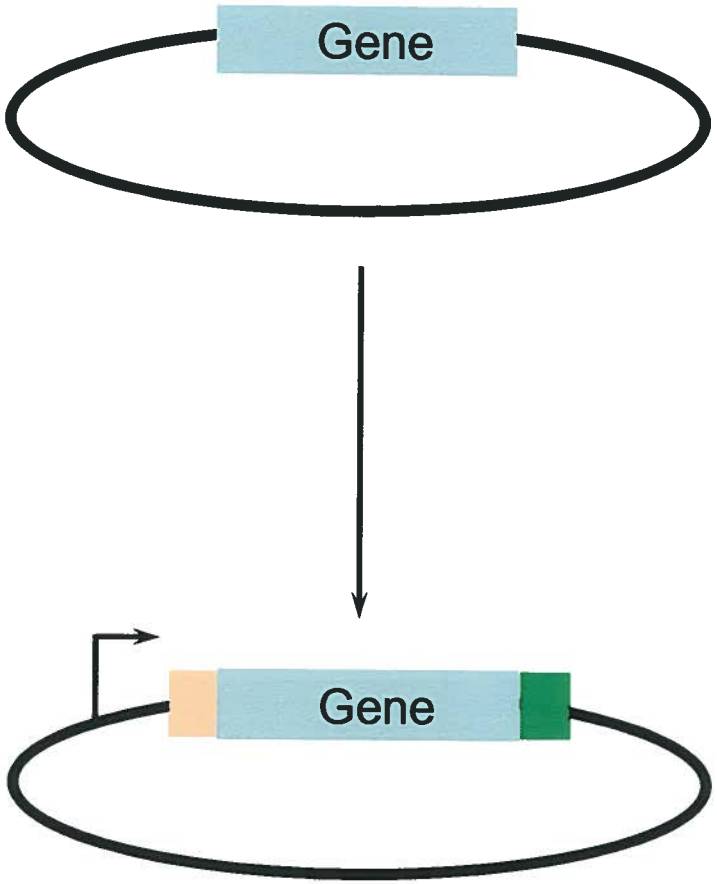
Mouse 101 Course, November 2009

Funded by NIH Contract HHSN261200800001E

SAIC® | Frederick

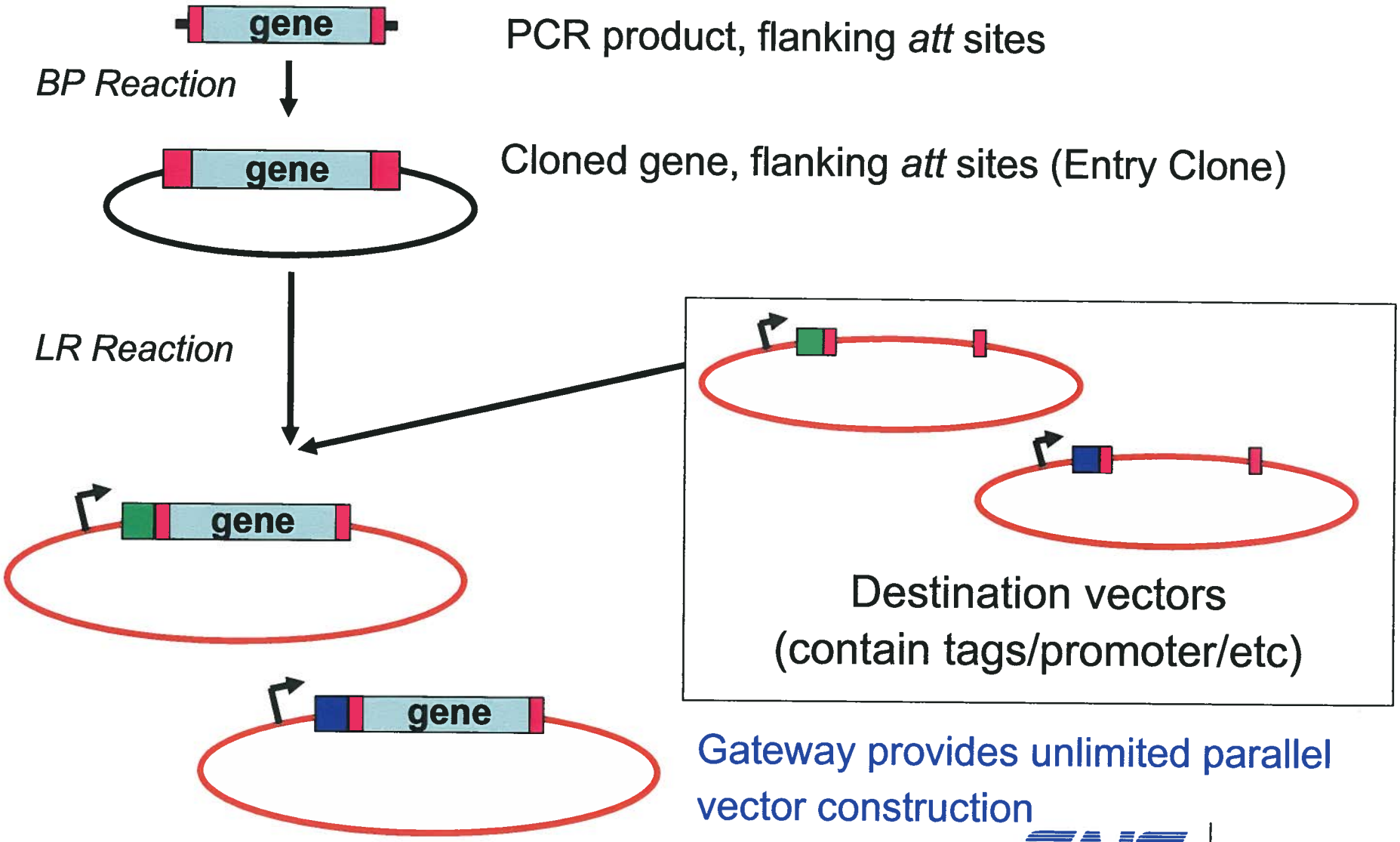
Designing a New Approach to Cloning

- Maximize flexibility
- Minimize planning
- Maintain reading frame
- Rapid
  - No restriction enzymes
  - No gel purification
  - No ligations
- High-throughput



SAIC® | Frederick

Gateway: Parallel Clone Construction

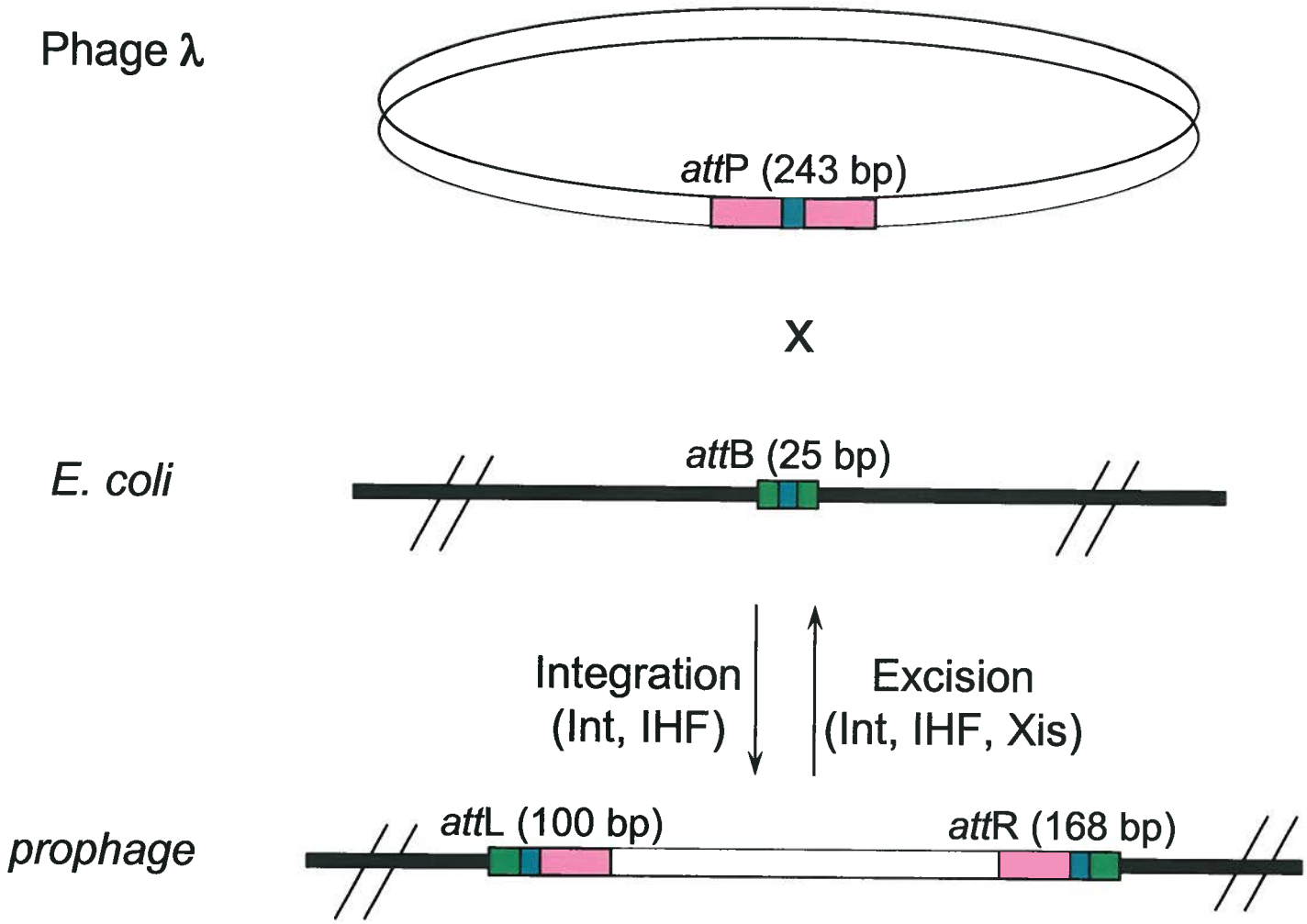


Gateway provides unlimited parallel vector construction

Final expression vectors

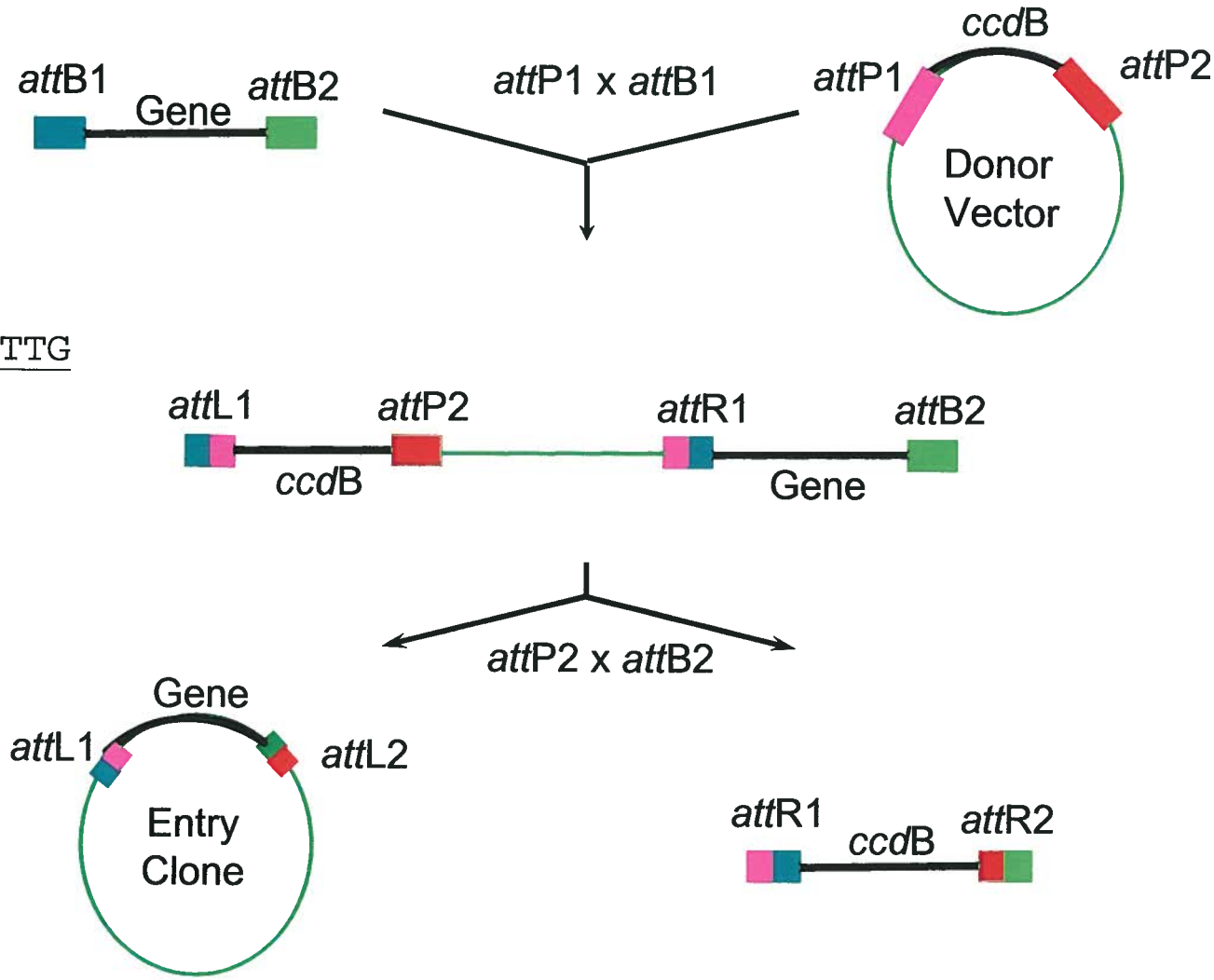
SAIC Frederick

Phage λ Recombination in E. coli



SAIC Frederick

Determinants of Specificity/Directionality



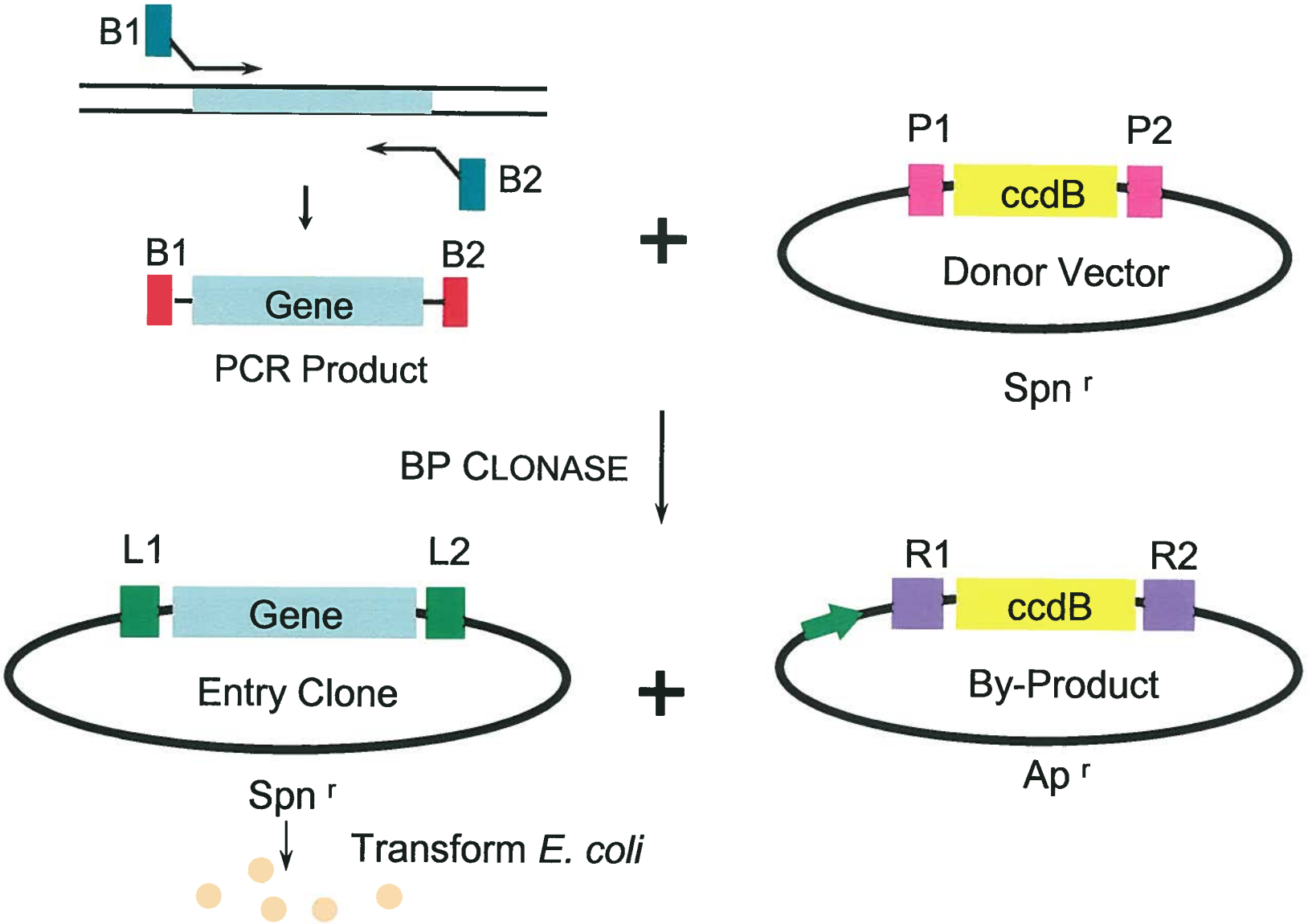
att site core sequence:

CAACTTTNNNNNNNAAAGTTG

att1: GTACAAA
att2: GTACAAG

SAIC Frederick

BP Reaction: Generating an Entry Clone



> 95% correct clones in Spn r colonies next day

SAIC | Frederick

Cloning PCR Products

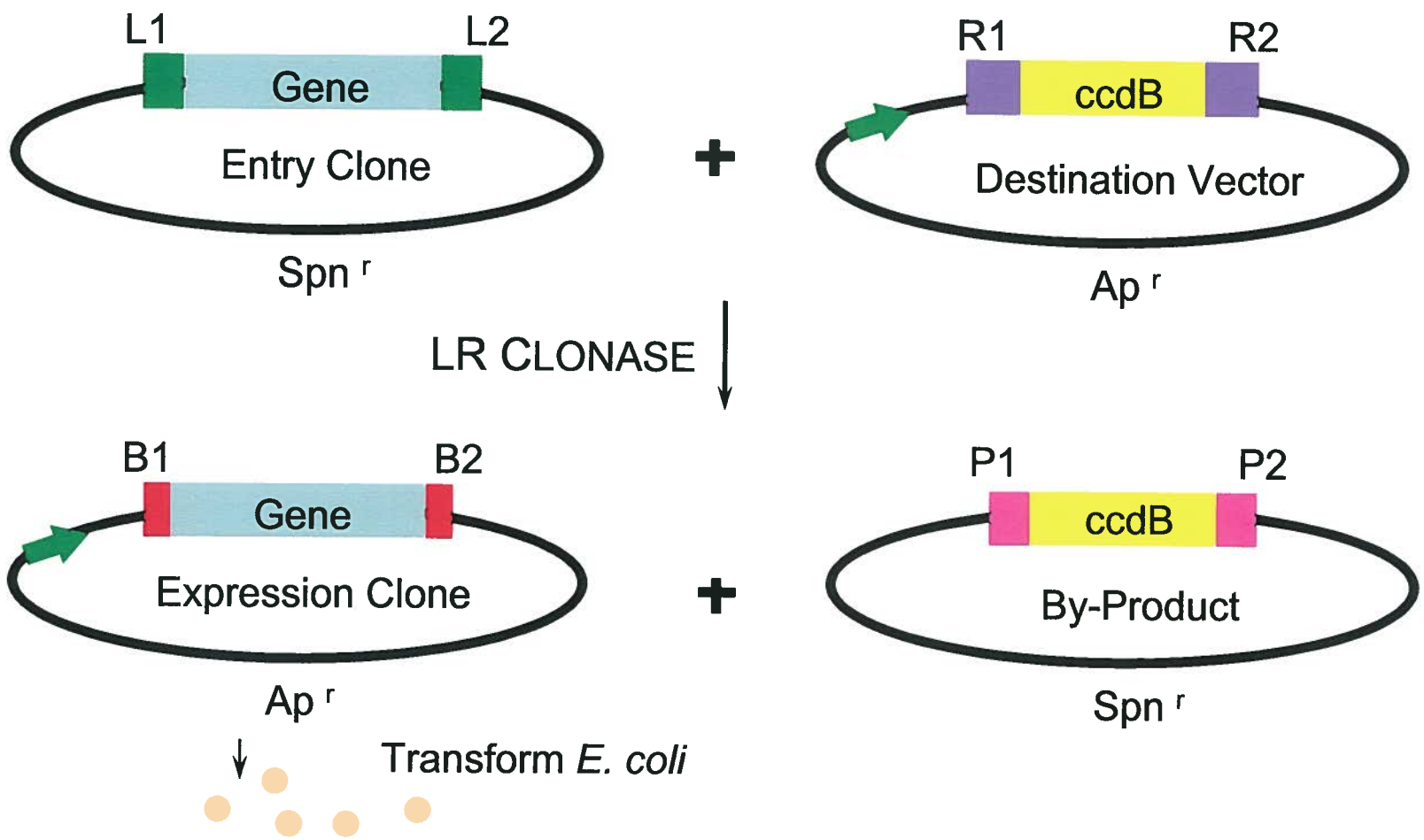
Size (kb)	PCR DNA (fmol)	PCR DNA (ng)	Colonies / ml Transformation	Correct Clones / Total Clones Examined
0.26	15	3	1223	10/10
	37.5	7.5	2815	
1.0	15	10	507	49/50
	37.5	25	1447	
1.4	15	14	271	48/50
	37.5	35	683	
3.4	15	34	478	9/10
	37.5	85	976	
4.6	15	46	190	10/10
	37.5	115	195	
6.9	15	69	30 (235^2)	47/50
	37.5	173	54 (463^2)	
10.1	7.5	50.5	16 (112^2)	15/16
	37.5	252.5	42 (201^2)	

SAIC® | Frederick

Where do Entry Clones come from?

- BP Recombination
  - Used to generate Entry clones from attB-containing PCR products
  - Requires BP Clonase and Donor Vector
  - Maximum flexibility for manipulating sequences
- Entry Vector Cloning
  - Entry Vectors (pEntr) can be used for REaL cloning
  - TOPO vectors available
  - Less flexibility in sequences at ends/junctions
- Library Clones
  - Many cDNA libraries use attB-containing vectors
  - human and mouse ORFeome in Entry Clones are available

LR Reaction: Generating Subclones



> 95% correct clones in Ap r colonies next day

SAIC Frederick

Obtaining Destination Vectors

- Invitrogen (limited options, high cost)
- Novagen (modified pET vectors)
- Addgene (numerous investigator constructed vectors)
- Clone Optimization Group (currently 300+ vectors)
- Make it yourself (using Invitrogen DV Construction Kit)

COG Destination Vectors

Destination vectors can include:

- Promoters (T7, CMV, AOX1, polyhedrin)
- Purification tags (His6, GST, FLAG, etc...)
- Solubility tags (MBP, NusA, Trx, etc...)
- Epitope tags (HA, myc, V5, etc...)
- Localization tags (eGFP, mRFP, etc...)
- Replication elements (origins, transactivators, terminators)
- Specialized signals (baculovirus tranposition, lentiviral LTRs)
- Protease cleavage sites (Tev, thrombin, etc...)
- Secretion signals (GP67, alpha mating factor)

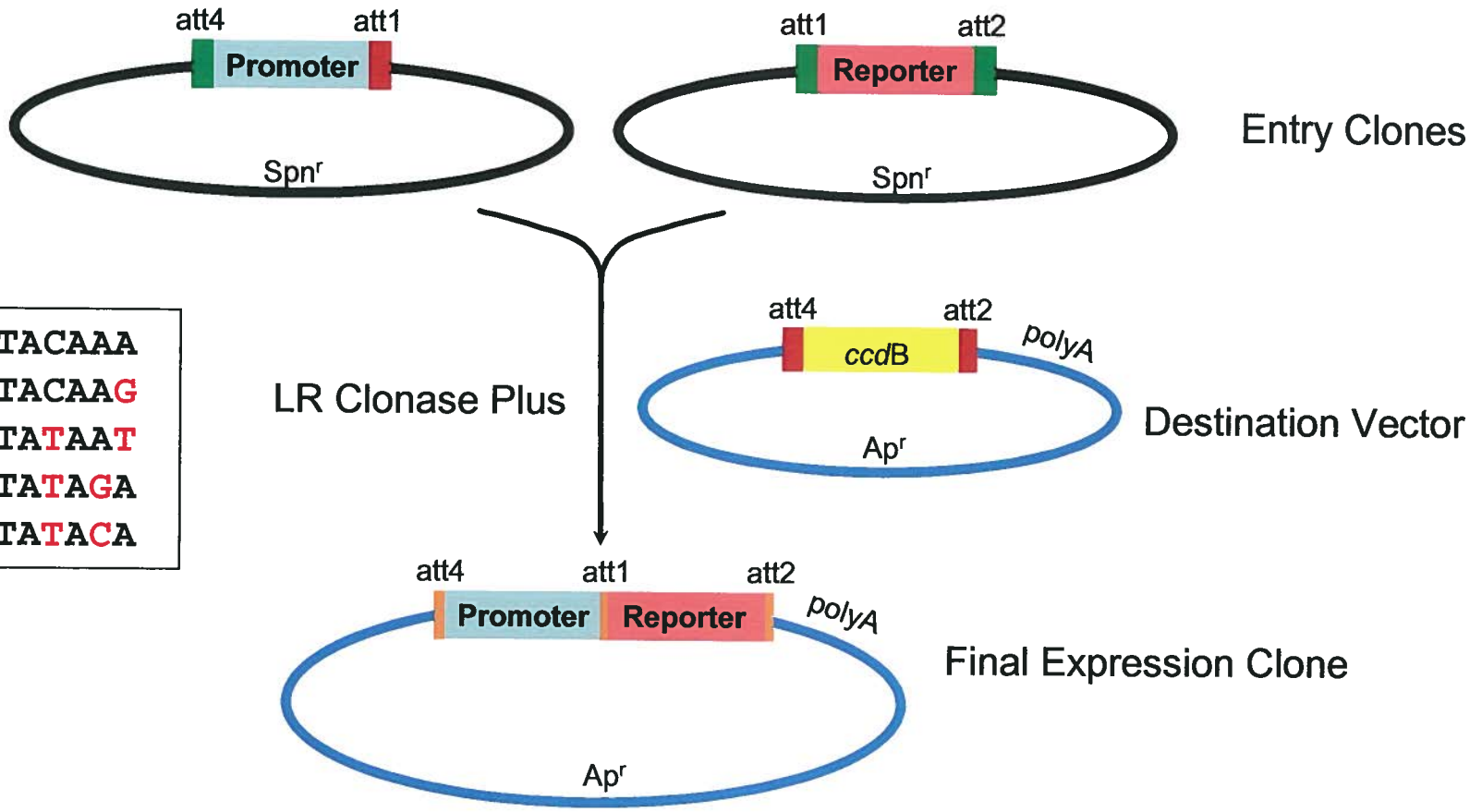
Standard Gateway Cloning Process

1) Construct design
2) Order oligonucleotides
3) PCR amplification/cleanup (day 1)
4) BP recombination/transformation (day 1)
5) Pick colonies, grow cultures (day 2)
6) Verify Entry clones (day 3)
7) Sequence verify Entry clones
8) LR recombination/transformation (day 4)
9) Pick colonies, grow cultures (day 5)
10) Verify Expression clones (day 6)

Best case: PCR to final expression clones in 6 days plus time to sequence—generally 2 weeks total time

SAIC® | Frederick

Multisite Gateway Recombination



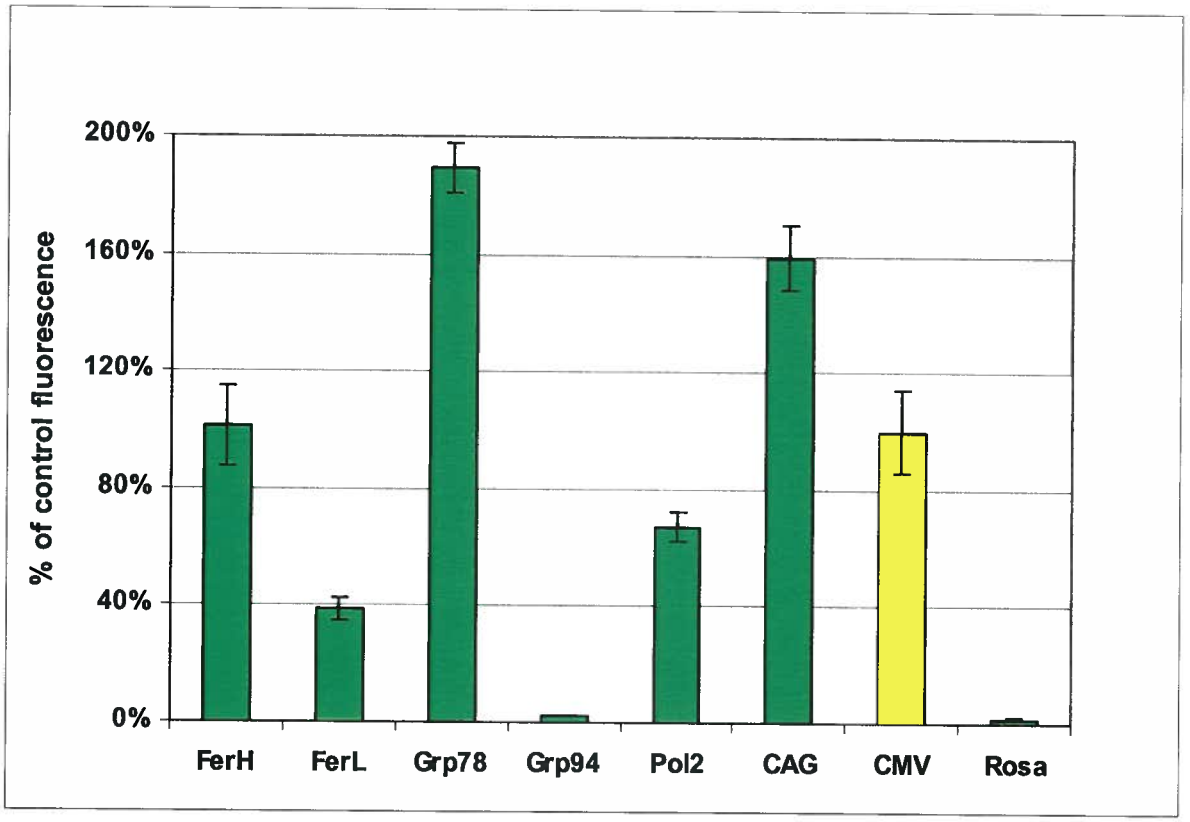

```
att1: GTACAAA
att2: GTACAAG
att3: GTATAAT
att4: GTATAGA
att5: GTATACA
```

Sequence verified Entry clones of promoters, reporters, or markers can be used to make large numbers of final expression constructs in parallel, and with completely identical vector backbones!

SAIC® | Frederick

Testing Alternative Promoters

293E attached
150,000 cells
200 ul volume
24 hrs



SAIC® | Frederick

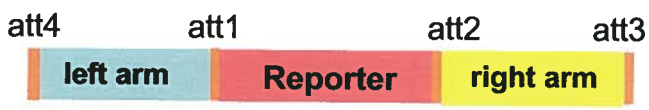
Multisite Gateway Formats

att4/att1/att2/att3 design: 3 component

For easier vector construction, element can be:

- IRES-FP
- promoter-FP
- selectable marker

shRNA constructs



homologous targetting constructs

SAIC® | Frederick

Multisite Gateway Formats

att4/att5/att1/att2 design: 3 component, gene right

Promoter libraries

- CMV
- FerH
- FerL
- Grp78
- UbC
- SV40
- PGK
- mRosa
- mPol2
- Alb
- Pbsn
- CMV-tet
- TRE-tight

UTR/Tag libraries

- HTLV RU5 LTR
- chEF1 LTR
- His6 fusion tag
- His6-eGFP fusion tag
- His6-MBP fusion tag

Note: no upper limit to number of fragments that can be recombined in a Multisite reaction!

SAIC Frederick

Reporter Constructs

- Fluorescent proteins
  - eGFP, eYFP, eCFP, BFP, CyPet, Venus
  - mOrange, DsRed2, DsRedExpress2, mRFP1, mCherry, mKate
  - paeGFP, pamCherry, mEos2, tdEos2
- Luminescence reporters
  - firefly luciferase (ffLuc2)
  - Renilla luciferase (rLuc)
- Multifunctional reporter fusions
  - ffLuc2-eGFP
  - ffLuc2-mRFP
  - rLuc-mRFP
  - ffLuc2-mCherry
  - ffLuc2-tdRFP2(12)

Destination Vectors for Mouse Work

- "Mammalian" expression vectors
  - usable for mammalian expression as well (cell lines for testing)
  - restriction enzyme sites at ends to remove transgenes
  - high copy number, robust vectors
- Lentiviral expression vectors
  - large number of backbone options
  - more details in Bruce Crise's talk to follow

Gateway Cloning Advantages

- Reduced need for sequencing
- Flexibility in changing out markers/elements
- Identity of plasmid backbones
- Ease of generating mutant clones
- High-throughput, rapid, relatively low cost
- Faster time-to-results!

Using PEL Services

- Protein Expression Core Services
  - requests made through CSAS online system, first come-first served
  - ODRS can subsidize 50% of cost of “risky” or “trial” projects
  - contact PEL group leaders for upfront estimate/discussions
- Clone Optimization Group
  - cloning/subcloning/DNA preparation/mutagenesis
  - deliverables include electronic reports, sequence files, plasmid DNA (mini or maxiprep)
  - can interface with LASP for construct delivery
- Viral Technology Group
  - lentivirus and adenovirus production
  - deliverables include concentrated and titered lentivirus or adenovirus

Acknowledgements

Clone Optimization Group

Leslie Garvey
Vanessa Wall
Jennifer Mehalko
Lauren Procter

Gateway R&D

Jim Hartley
Mike Brasch
David Cheo
Devon Byrd

SAIC® | Frederick