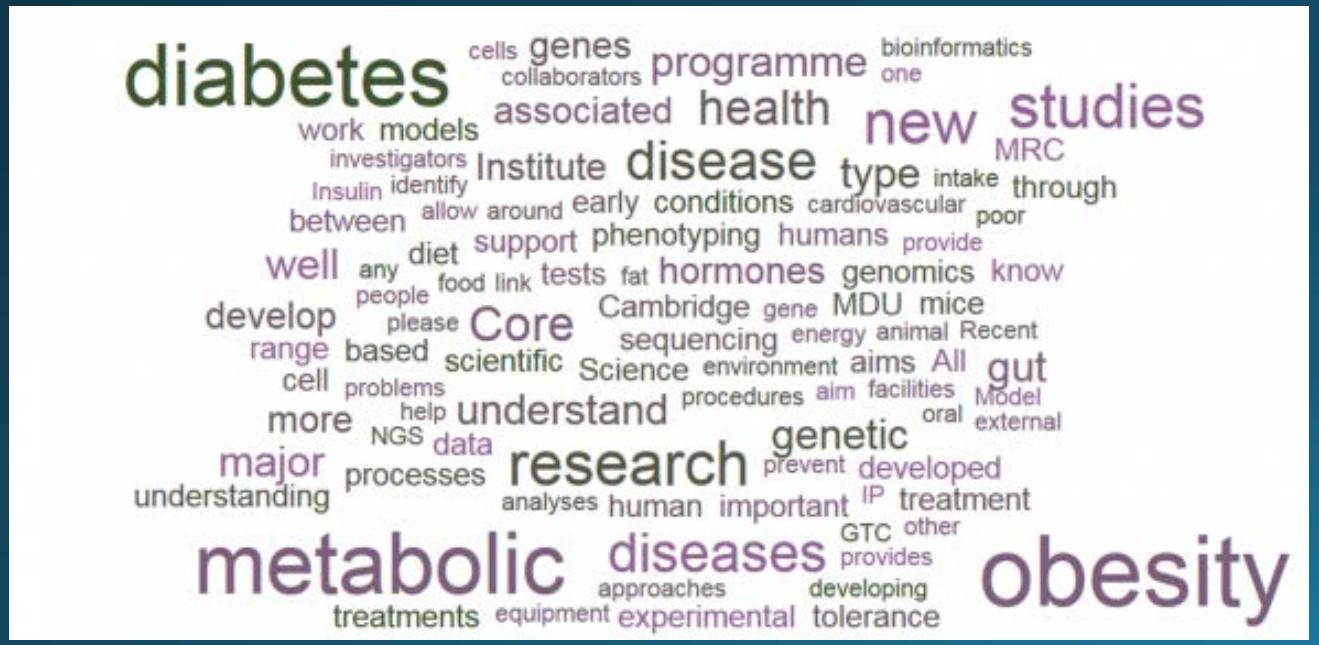
Using IVA cloning to generate complex constructs to study metabolic diseases in stem cell models

MSc. Onur Temocin

22.11.2018



SCIENTIFIC REPORTS

OPEN

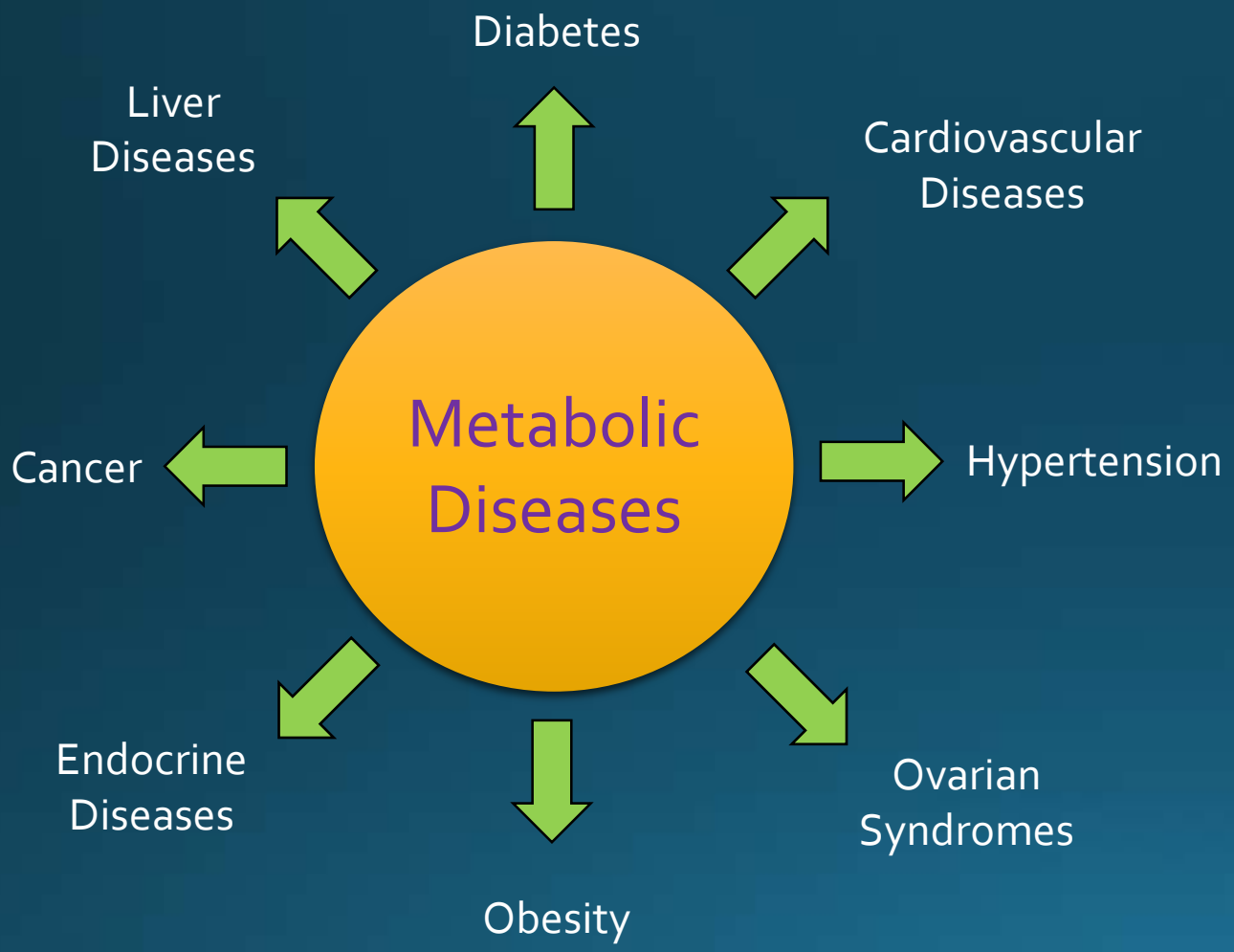
IVA cloning: A single-tube universal cloning system exploiting bacterial *In Vivo* Assembly

Received: 22 March 2016
Accepted: 13 May 2016
Published: 06 June 2016

Javier García-Nafría*, Jake F. Watson* & Ingo H. Greger

In vivo **homologous recombination holds the potential for optimal molecular cloning, however, current strategies require specialised bacterial strains or laborious protocols. Here, we exploit a recA-independent recombination pathway, present in widespread laboratory *E.coli* strains, to develop IVA (*In Vivo* Assembly) cloning. This system eliminates the need for enzymatic assembly and reduces all molecular cloning procedures to a single-tube, single-step PCR, performed in <2 hours from setup to transformation. Unlike other methods, IVA is a complete system, and offers significant advantages over alternative methods for all cloning procedures (insertions, deletions, site-directed mutagenesis and sub-cloning). Significantly, IVA allows unprecedented simplification of complex cloning procedures: five simultaneous modifications of any kind, multi-fragment assembly and library construction are performed in approximately half the time of current protocols, still in a single-step fashion. This system is efficient, seamless and sequence-independent, and requires no special kits, enzymes or proprietary bacteria, which will allow its immediate adoption by the academic and industrial molecular biology community.**

What are metabolic diseases?



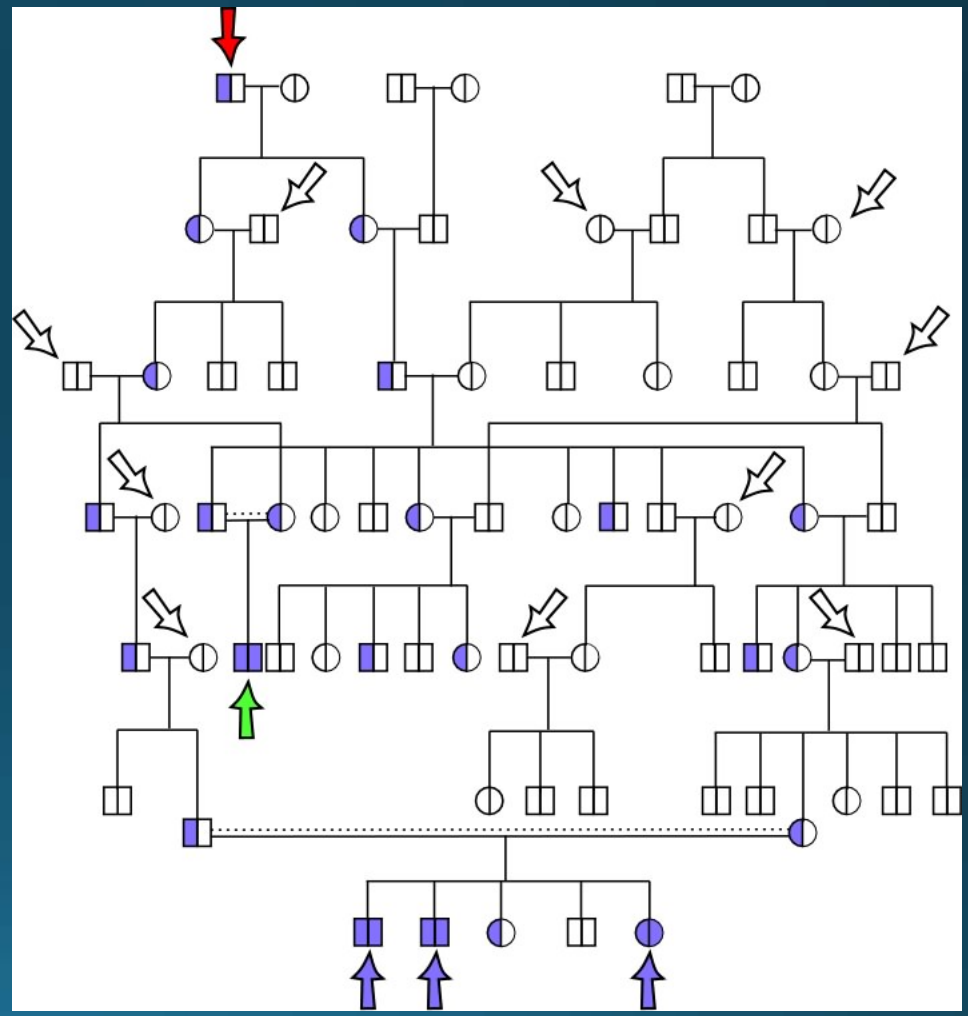
Contributing factors:

- Lifestyle and diet
- Exposure to pollutants
- Genetic background

Pitsavos et al. 2006
Ruzzin et al. 2012
Heindel et al. 2017
Battistoni et al. 2018

Human studies are limited

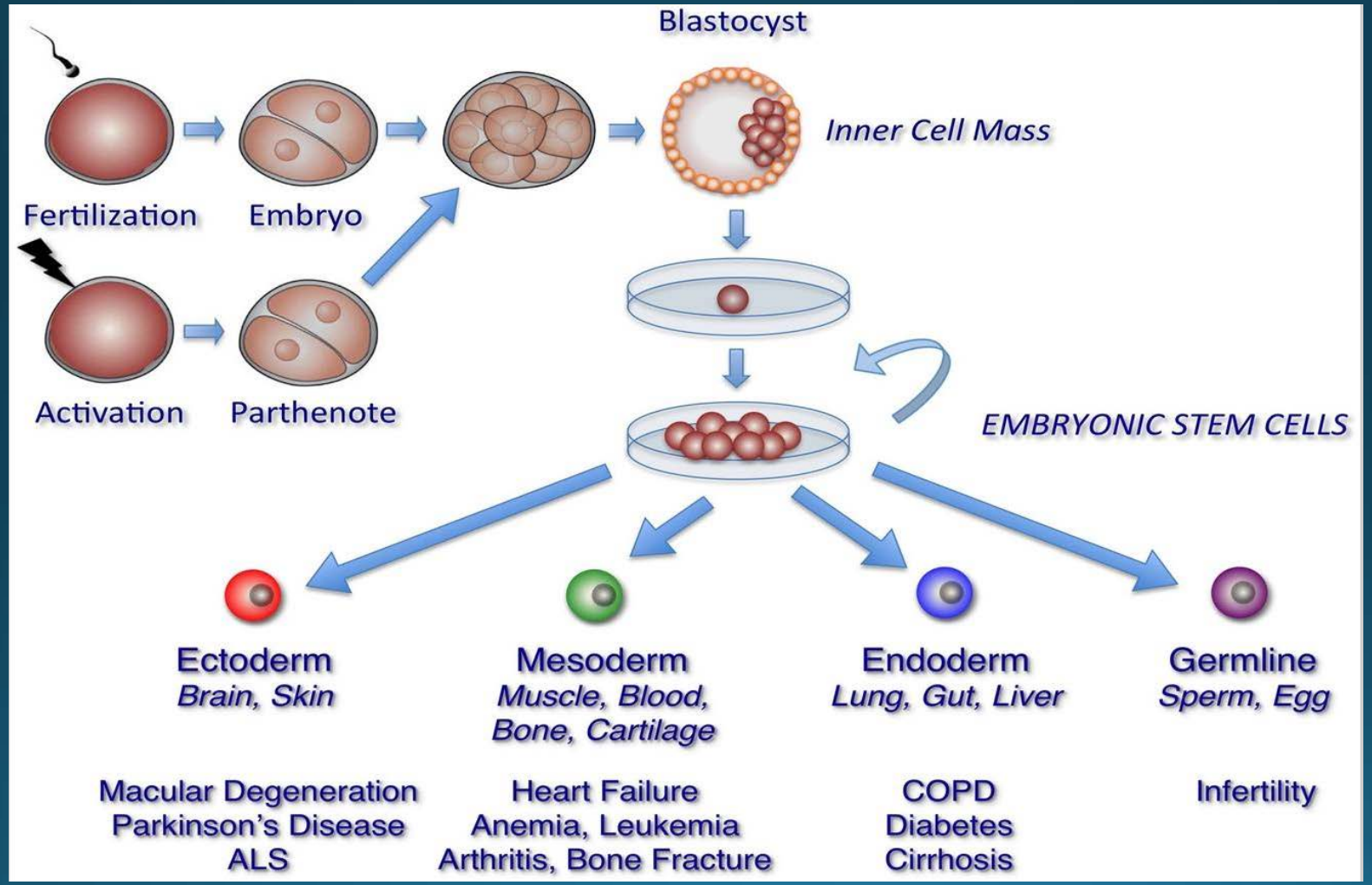
- Restricted access to human samples
- Genetic variations and family history
- Case studies are incomplete



IUB Indiana

Generating any cell type of the body

Embryonic stem cells



Yabut&Bernstein 2011

Limited access

More difficult conditions for culturing

Manipulation methods are inefficient

How to study gene function?

Genome editing approaches to create:

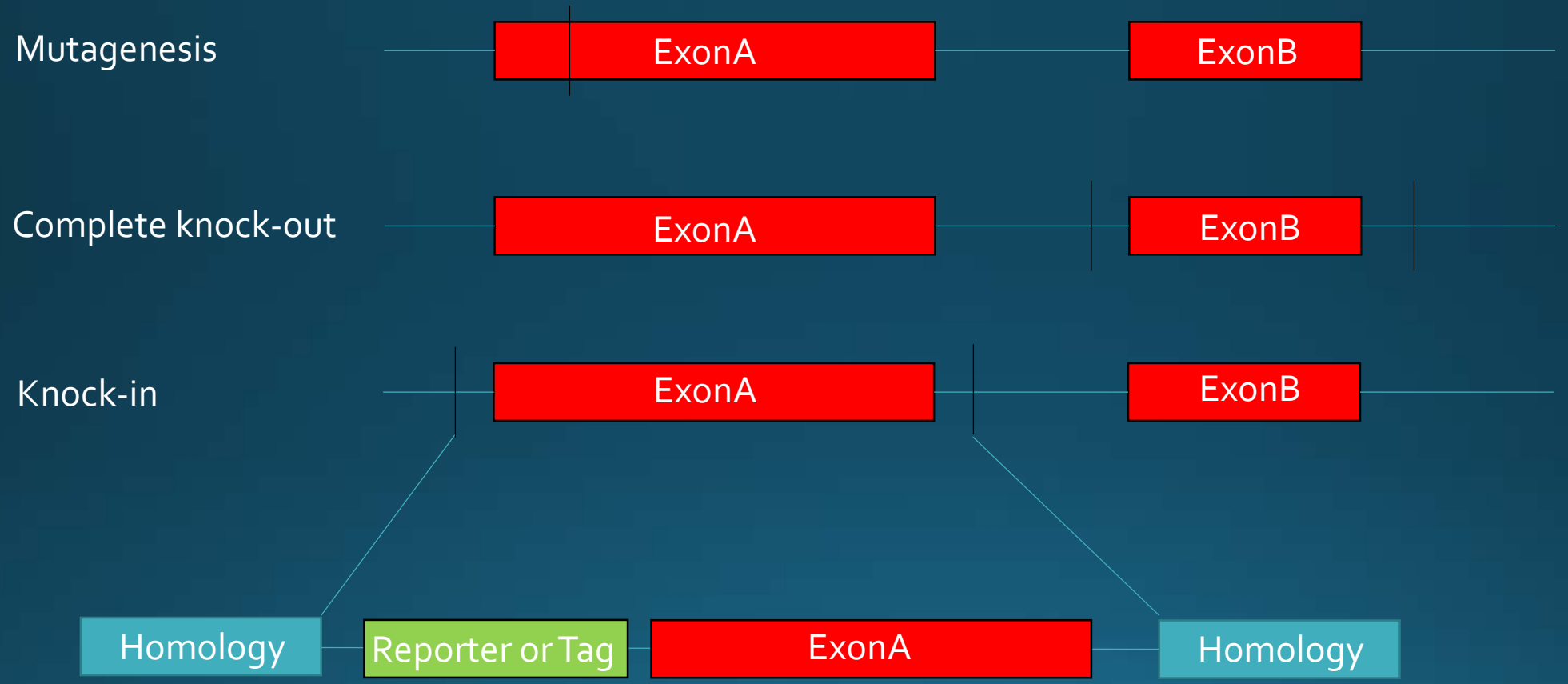
- Gain- or loss-of-function studies
- Gene overexpression for transcriptome analysis
- Tools to investigate protein-protein interactions

clinicalleader.com

Maeder et al. 2016

Zhang et al. 2017

Construct design for efficient gene targeting



Zhang et al. 2013
Vidigal et al. 2015
Acosta et al. 2018

Limitations in genome targeting to study metabolic diseases

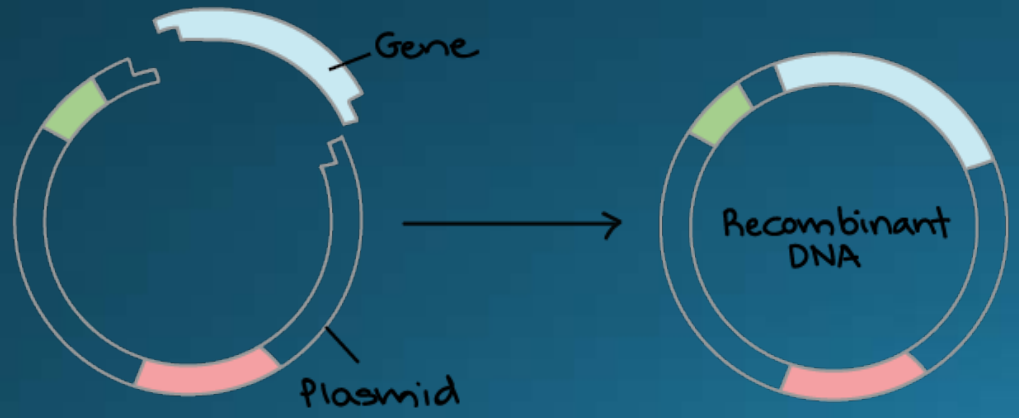
Current limitations in all strategies:

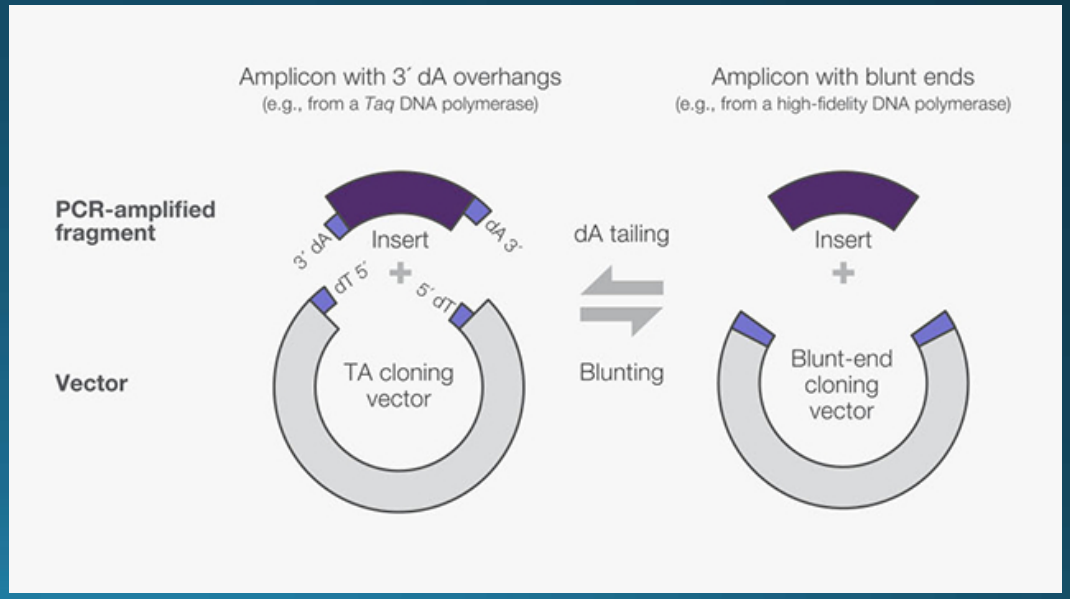
- ✓ Generation of large plasmids
- ✓ Efficiency of gene targeting
- ✓ Screening methods
- ✓ Efficiency of *in vitro* differentiation (mixed cell types)

bsscommerce.com

Generation of plasmids for gene targeting

- Classical cloning (Digest-ligate system with restriction enzymes)
- Blunt and TA cloning (Using A tailing ability of Taq polymerase)
- Synthetic DNA fragment design (oligo assembly)
- **IVA (In vitro assembly)**

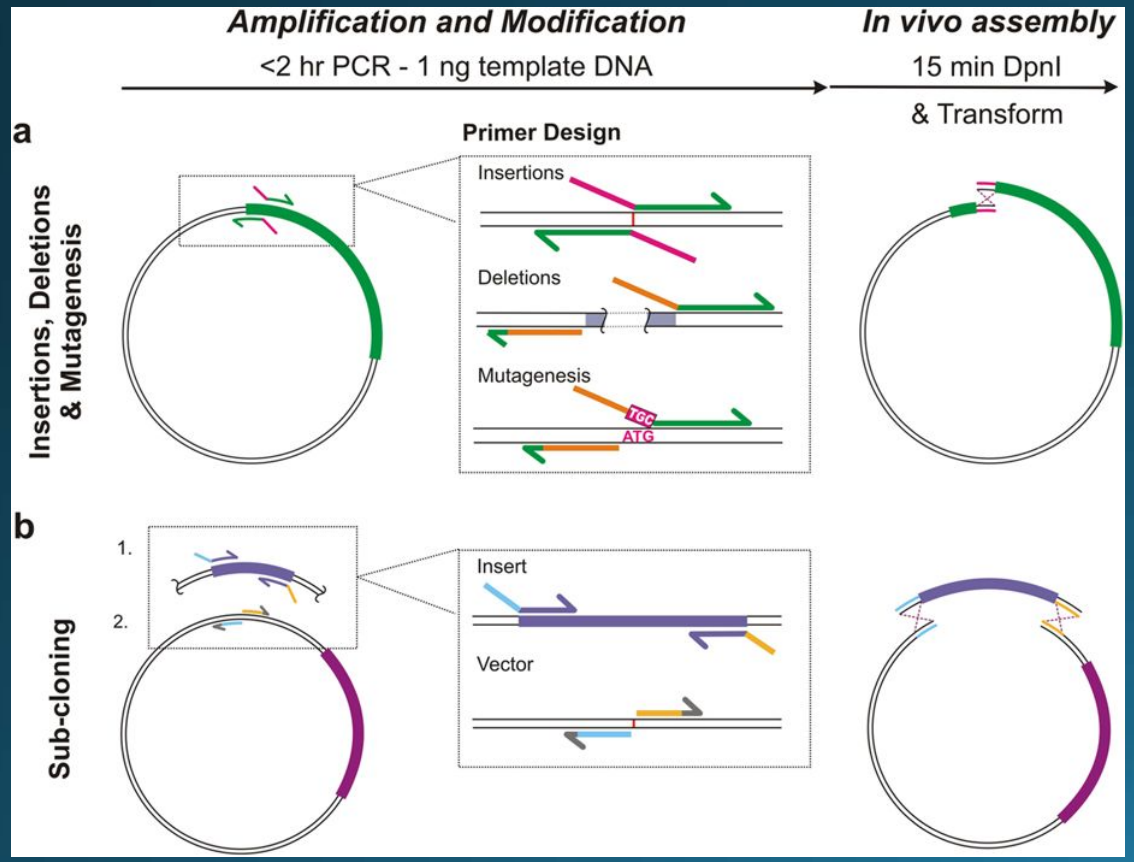


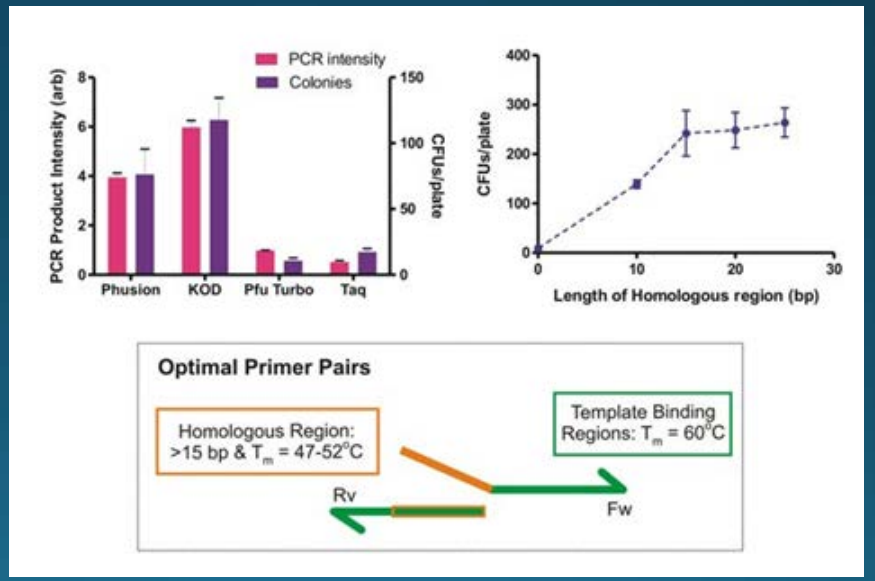


Thermo Fisher Scientific

IVA cloning for higher plasmid complexity (Garcia et al. 2016)

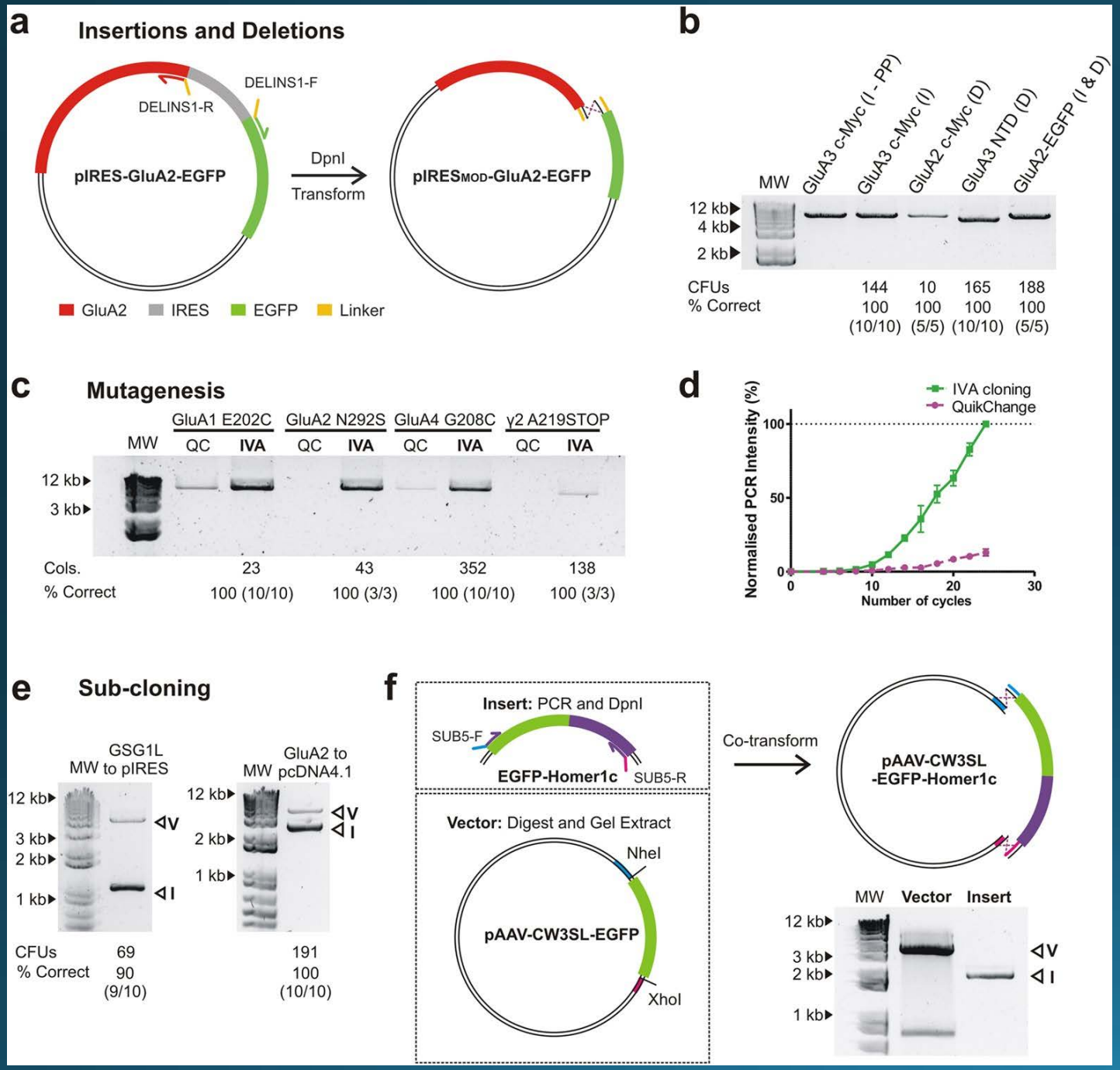
Design Primers → PCR amplify and purify fragments → Transform competent bacteria with the fragments → Incubate bacteria in SOC medium and plate on agar → Screen agar plate bacteria by colony PCR → Expand relevant colonies for plasmid preparation

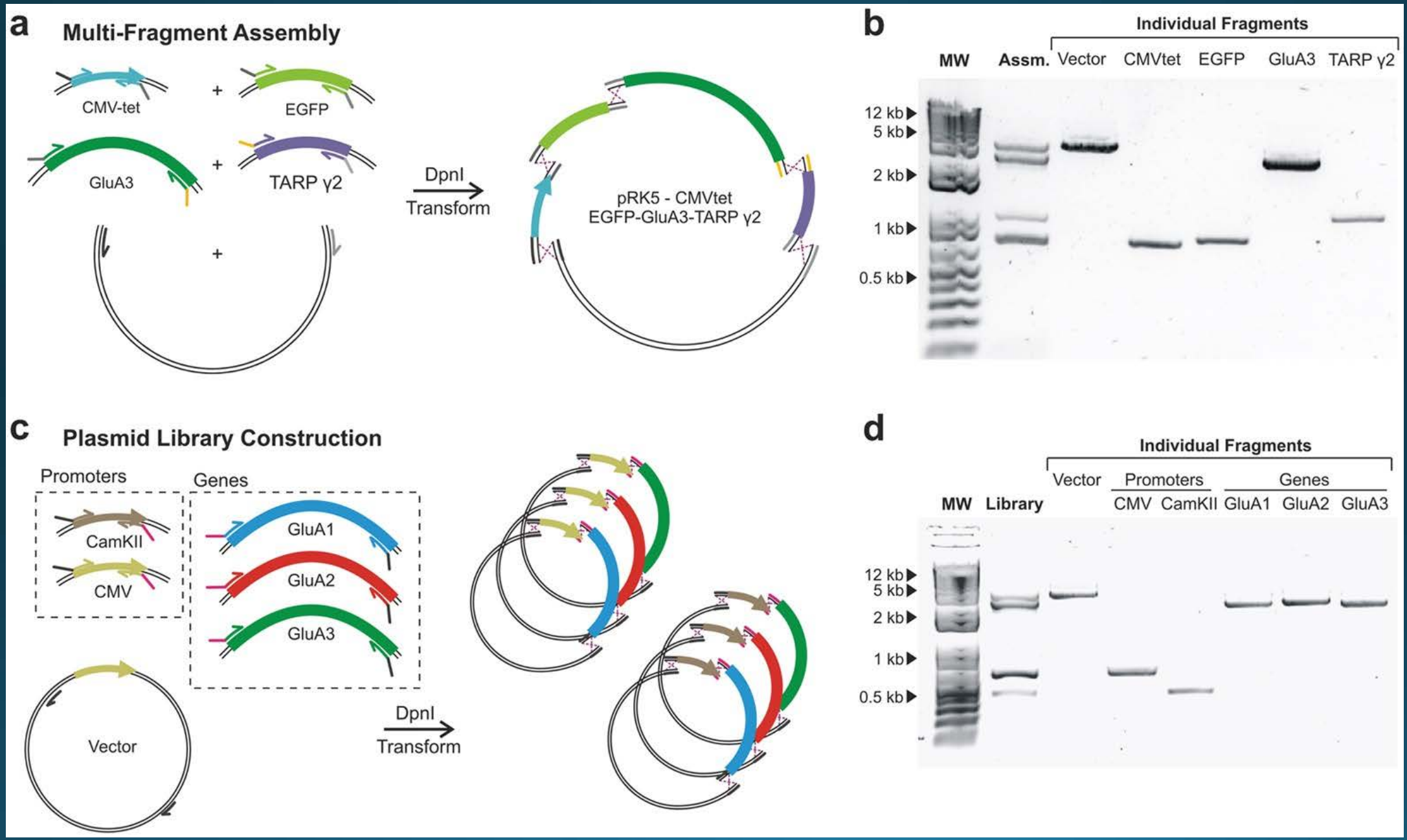




Advantages

1) Time & cost efficient
2) Flexibility
3) No dependency on restriction enzymes


a
Insertions and Deletions
DELINS1-F
DELINS1-R
pIRES-GluA2-EGFP
DpnI
Transform
pIRESMOD-GluA2-EGFP
GluA2
IRES
EGFP
Linker
b
MW
GluA3 c-Myc (I - PP)
GluA3 c-Myc (I)
GluA2 c-Myc (D)
GluA3 NTD (D)
GluA2-EGFP (I & D)
12 kb
4 kb
2 kb
CFUs
% Correct
144
100
(10/10)
10
100
(5/5)
165
100
(10/10)
188
100
(5/5)
c
Mutagenesis
GluA1 E202C
GluA2 N292S
GluA4 G208C
γ2 A219STOP
MW
QC
IVA
12 kb
3 kb
Cols.
% Correct
23
100 (10/10)
43
100 (3/3)
352
100 (10/10)
138
100 (3/3)
d
IVA cloning
QuikChange
Normalised PCR Intensity (%)
Number of cycles
e
Sub-cloning
GSG1L
MW to pIRES
GluA2 to
MW pcDNA4.1
12 kb
3 kb
2 kb
1 kb
V
I
CFUs
% Correct
69
90
(9/10)
191
100
(10/10)
f
Insert: PCR and DpnI
SUB5-F
EGFP-Homer1c
SUB5-R
Vector: Digest and Gel Extract
NheI
pAAV-CW3SL-EGFP
XhoI
Co-transform
pAAV-CW3SL
-EGFP-Homer1c
MW
Vector
Insert
12 kb
3 kb
2 kb
1 kb
V
I
a
Moving position of an epitope tag
INS3-F
INS3-R
pCustom-GluA2-
C-terminal-FLAG
DEL3-R
DEL3-F
DpnI
Transform
pCustom-GluA2-
N-terminal-FLAG
(1)
(2)
FLAG
GluA2
b
MW
5 kb
2 kb
1 kb
(1)
(2)
CFUs
% Correct
273
100 (5/5)
c
Combining Multiple Modifications
SUB3-F
SUB3-R
Insert: TARP γ2
DELINS2-R
DELINS2-F
pRK5 - GluA3
SUB4-R
SUB4-F
DpnI
Transform
pRK5 -FLAG-GluA3
ΔNTD-GSGSG-TARP γ2
(1)
(2)
(3)
TARP γ2
GluA3
GluA3 NTD
GSGSG Linker
FLAG
d
MW
5 kb
2 kb
1 kb
(1)
(2)
(3)
CFUs
% Correct
43
70 (7/10)
e
Multi-site potential of IVA cloning
[1] - MUT9
[2] - MUT10
[3] - MUT11
[4] - MUT12
[5] - MUT13
DpnI
Transform
f
Modifications
MW
[1]
[1-2]
[1-3]
[1-4]
[1-5]
12 kb
2 kb
1 kb
0.5 kb
g
Colonies Formed
% Colonies Correct
CFUs/plate
% Colonies Correct
Number of modifications


a
Multi-Fragment Assembly
CMV-tet
EGFP
GluA3
TARP γ2
DpnI
Transform
pRK5 - CMVtet
EGFP-GluA3-TARP γ2
b
Individual Fragments
MW
Assm.
Vector
CMVtet
EGFP
GluA3
TARP γ2
12 kb
5 kb
2 kb
1 kb
0.5 kb
c
Plasmid Library Construction
Promoters
Genes
CamKII
CMV
GluA1
GluA2
GluA3
Vector
DpnI
Transform
d
Individual Fragments
Vector
Promoters
Genes
MW
Library
CMV
CamKII
GluA1
GluA2
GluA3
12 kb
5 kb
2 kb
1 kb
0.5 kb

An efficient cloning for regenerative studies

- Unlimited material and datasets (compared to case studies)
- Better understanding of regulatory networks and differentiation
- Boosting of cure development process in regenerative medicine

Allied academics Amsterdam

THANK YOU FOR YOUR ATTENTION!!