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Protocol

The StarGate system

Two-Step combinatorial cloning system

1 DESCRIPTION

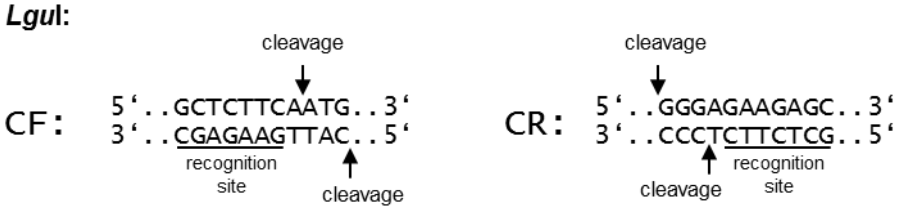
StarGate has been developed for the rapid systematic screen of the optimal expression and purification system for a given gene of interest (GOI). It offers a high cloning efficiency due to the directed insertion of the GOI in a two-step approach. In the first step, the GOI is cloned into a donor vector from which it can be subcloned by a standardized procedure into a variety of acceptor vectors. Acceptor vectors provide different genetic surroundings without the need for sequencing each individual vector. Due to the short combinatorial sites, the modification the GOI are minimal. The generated final expression vector is placed into the respective host.

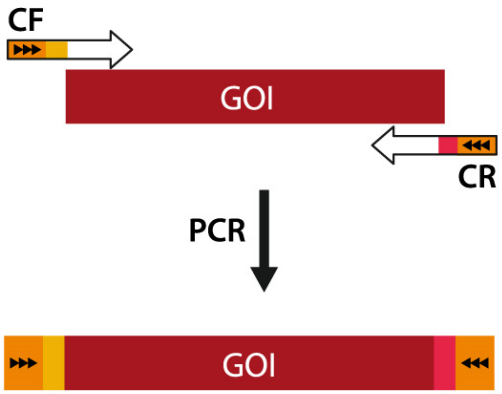
In this protocol we describe the generation of destination vectors for the expression of one or more proteins from a single vector. For a direct transfer into an acceptor vector, use the StarGate direct transfer protocol.

2 GENERAL INFORMATION AND NECESSARY COMPONENTS

2.1 Donor vector generation

For the generation of the donor vector, the GOI has to be equipped with the integration sites at both termini via polymerase chain reaction (PCR). The integration sites consist of the *Lgu*I recognition sites and a 4 base compromising combinatorial site. *Lgu*I is a type IIS restriction enzyme that cleaves the DNA double strand outside the recognition site (see scheme below). The *Lgu*I recognition site, as well as the combinatorial sites are introduced by the forward (CF) and reverse (CR) primers. Thereby, the digestion with only one single enzyme can generate two different independent sticky ends with 3-bases 5'-overhangs. The different combinatorial sites lead to the oriented insertion of the PCR fragment into the entry vector, pENTRY-IBA51. In addition, after digestion the recognition sequence is removed completely. Therefore, the encoded amino acid sequence is not affected by remaining restriction enzyme sites, enabling the expression of authentic proteins.

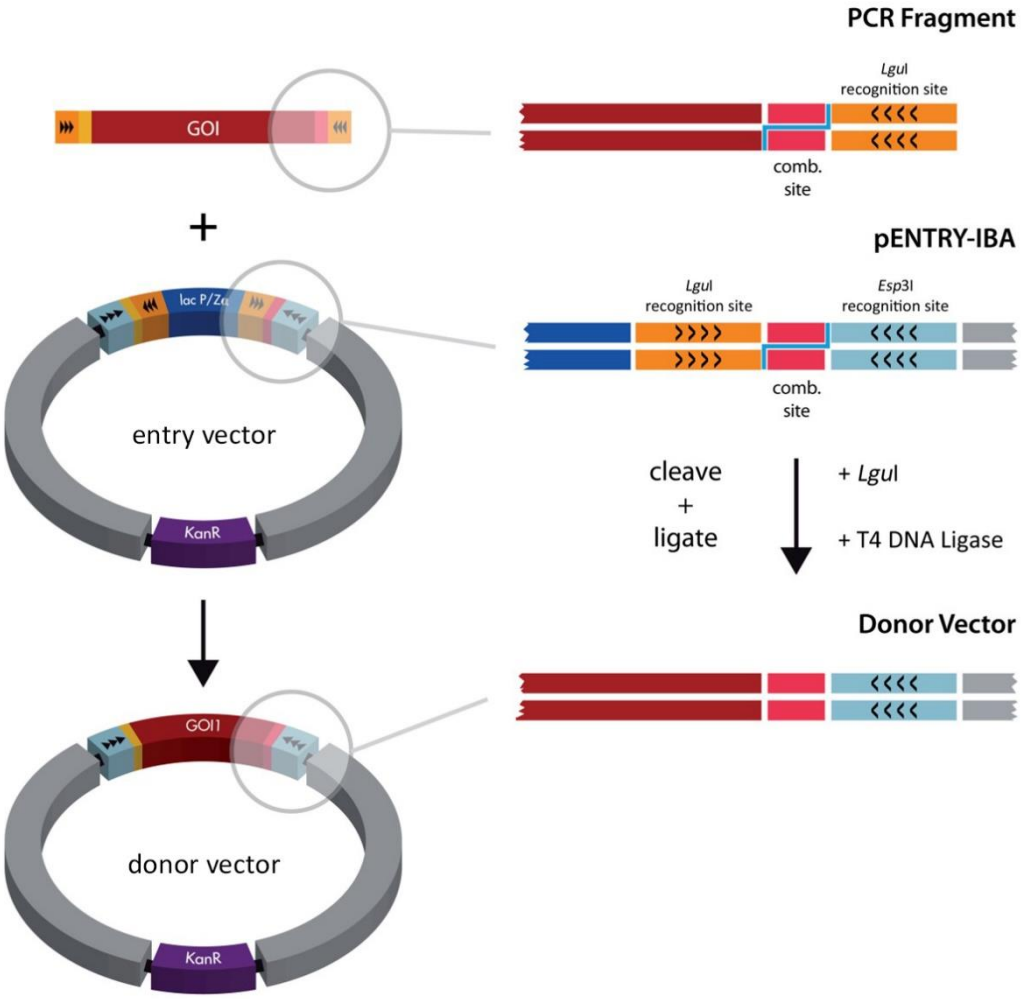




In this schematic representation of PCR product generation.
The forward primer (CF) starts with a sequence containing the *Lgu*I recognition site (GCTCTTC; orange with arrows indicating its orientation) followed by the downstream AATG combinatorial site. The start codon ATG is already included. The primer sequence continues with nucleotides complementary to the antisense strand of the GOI.

The reverse primer (CR) equally starts with a sequence containing the *Lgu*I recognition site (GCTCTTC) followed by TCCC (the reverse complement of the downstream combinatorial site GGGA), which again is directly followed by a sequence which is reverse complementary to the 3'-end of the GOI.

The PCR product can then be used in a one-step reaction, where *Lgu*I restriction enzyme cleaves both it and the entry vector. Due to the different combinatorial sites, the PCR product is directionally ligated into the entry vector. The same combinatorial sites in the resulting donor vector are now flanked by *Esp*3I recognition sites, enabling a highly efficient and specific GOI transfer process into correspondingly designed acceptor vectors. Insertion of PCR product/GOI into the donor vector can be checked by restriction analysis. However, as PCR may lead to mutations and to improper product ends, it is recommended to confirm GOI and flanking sequences by sequencing.



The insertion of GOI into the entry vector pENTRY-IBA51 results in a donor vector. *Lgu*I cleaves next to its recognition site, creating overhangs which are named combinatorial sites. T4 DNA ligase generates the final donor vector. The PCR product and the entry vector are recombined at the combinatorial sites (red and yellow), accompanied by loss of the *Lgu*I recognition sites (orange). The same combinatorial sites are flanked by *Esp*3I recognition sites (blue) in the resulting donor vector, enabling GOI transfer into acceptor vectors.

2.1.1 Example for the primer design

In case of using a proofreading polymerase, which is highly recommended (e.g., *Pfu*), 3'-phosphorothioate protected primers should be used. Otherwise, proof reading activity may degrade the primers from the 3'-end during PCR, impairing annealing and consequently reduces the yield of PCR product.

Essential parameters for PCR optimization are the annealing temperature, the duration of synthesis and the template concentration. Initial hybridizing regions of primers should have a theoretical melting temperature between 60 °C and 63 °C. The primer melting temperatures can be derived by adding the single base melting temperatures of consecutive bases using 4 °C for each GC pairing and 2 °C for each AT pairing (and 1 °C for each GT pairing). The annealing temperature should be chosen at least 5 °C below the melting temperature of each primer. The number of cycles should be kept as low as possible to minimize base substitutions.

The GOI has the following sequence (leave out the Met start codon and the stop codon):

```
5'-TTGACCTGCAACAGCTGCATAGCC-3'
3'-AACTGGACGTTGTCGACGTATCGG-5'
   LeuThrCysAsnSerCysIleAla
```

Appropriate primers have to be designed so that the resulting PCR product will additionally include the combinatorial sites (bold and italic), the *Lgu*I recognition site (underlined) and 4 additional bases for efficient restriction enzyme activity. In this example, the resulting PCR product has the following sequence:

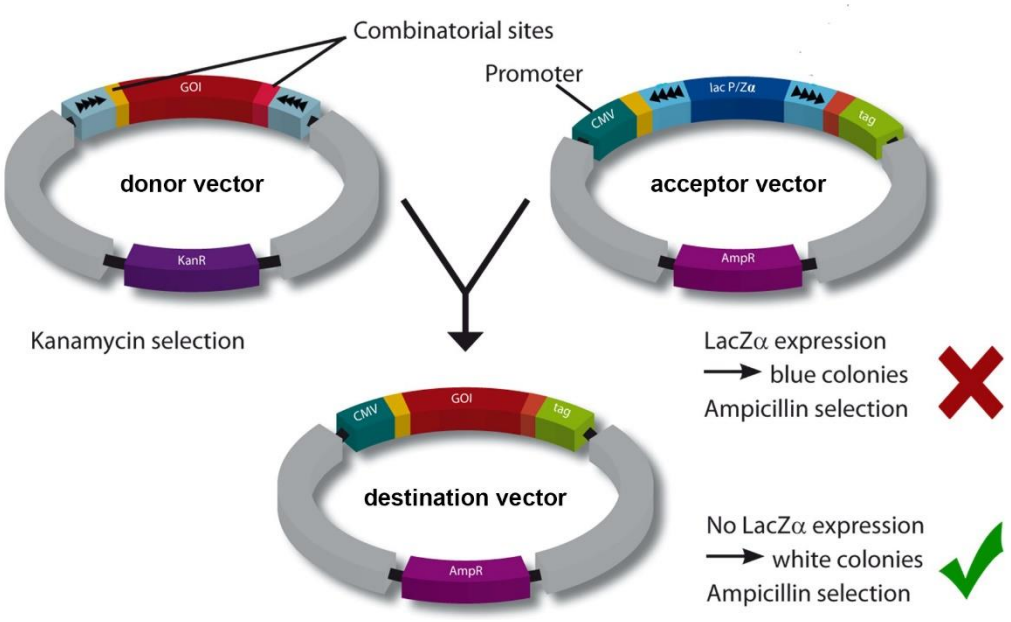
```
5'-AGCGGCTCTTCAATGTTGACCTGCAACAGCTGCATAGCCGGGAGAAGAGCCGCT-3'
3'-TCGCCGAGAAGTTACAACTGGACGTTGTCGACGTATCGGCCCTCTTCTCGGCGA-5'
      MetLeuThrCysAsnSerCysIleAlaGly
```

The Met start codon is reconstituted by the upstream combinatorial site and the stop codon is replaced by a glycine "GGG" codon included in the downstream combinatorial site to allow C-terminal fusions of the GOI.

The obtained PCR product is inserted into the entry vector pENTRY-IBA51, converting it into a donor vector.

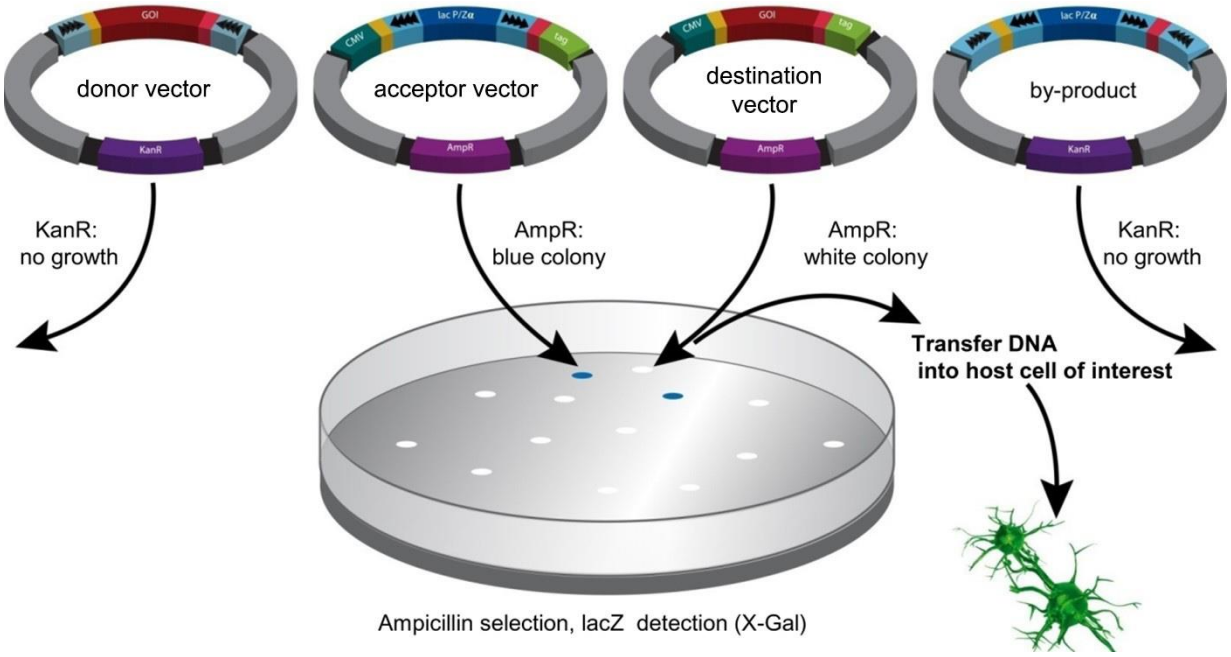
2.2 Destination vector generation

The final expression product, called the destination vector is generated by the transfer of the GOI from the donor vector into a selected acceptor vector. Acceptor vectors provide the different genetic surroundings (i.e., purification tag, promoter, signal sequence, etc.). The destination vector is formed by mixing the donor vector with the respective acceptor vector in a further one-tube reaction.



During the generation of the destination vector, recombination will take place at the combinatorial sites (red and orange), necessitating the presence of these sites in the destination vector. The more complex recognition sites are eliminated. Loss of the recognition sites drives the reaction towards generation of the desired destination vector.

E. coli is transformed with the mixture that potentially includes all 4 possible vector events (donor vector, acceptor vector, destination vector and by-product) and plated on LB agar plates containing ampicillin and X-gal. Desired destination vectors including GOI will generate white colonies while acceptor vectors without GOI will generate blue colonies.nan



Due to selection on ampicillin plates, donor vector and by-product – which provide a kanamycin resistance only – will not enable growth of *E. coli*. Acceptor vector and destination vector enable growth due to the encoded ampicillin resistance genes. The acceptor vector without GOI carries the LacZα gene and therefore produces blue colonies on X-gal containing plates. LacZα has been replaced by GOI in the destination vector, therefore generating white colonies.

2.3 Necessary components

Products by IBA	Cat. No.
pENTRY-IBA51 (provided as 5 µg aliquot (250 ng/µl))	5-4091-001
Competent cells *E. coli* Top10 (100 µl)	5-1600-020
Acceptor vector (provided as 5 µg aliquot (250 ng/µl))	Cat. No. listed under section 4

Enzymes and buffers	
dNTP Mix	Containing dATP, dTTP, dGTP, dCTP
Pfu DNA Polymerase and supplied buffer	
T4 DNA Ligase	Concentration 1 U/µl
DTT	Often provided with T4 DNA Ligase
ATP	Often provided with T4 DNA Ligase
*Lgu*I restriction enzyme and supplied buffer	Concentration 5 U/µl. The enzyme must be active at 37 °C.
*Esp*3I restriction enzyme and supplied buffer	Concentration 10 U/µl. The enzyme must be active at 37 °C. Do not use the isoschizomer *BsmB*I

Sequencing Primers (HPLC purified)	
5'- GCGAAACGATCCTCGAAGC -3'	Forward sequencing primer for pENTRY-IBA51
5'- CCCCTGATTCTGTGGATAACCG -3'	Reverse sequencing primer for pENTRY-IBA51
5'-GAGTTATTTTACCACTCCCT-3'	Forward sequencing primer for pASG-IBA and pASK-IBA vectors (*E. coli* vectors)
5'-CGCAGTAGCGGTAAACG-3'	Reverse sequencing primer for pASG-IBA and pASK-IBA vectors (*E. coli* vectors)
5'-GAGAACCCACTGCTTACTGGC-3'	Forward sequencing primer for pESG-IBA, pCSG-IBA, and pDSG-IBA vectors (mammalian vectors)
5'-TAGAAGGCACAGTCGAGG-3'	Reverse sequencing primer for pESG-IBA, pCSG-IBA, and pDSG-IBA vectors (mammalian vectors)

Additional material
LB agar plates with 50 mg/l kanamycin and 50 mg/l X-gal
LB agar plates with 100 mg/l ampicillin and 50 mg/l X-gal

3 PROTOCOL

3.1 PCR amplification of the GOI with *Pfu* polymerase

The following protocol is based on standard protocols for *Pfu* polymerase PCR.

3.1.1 Mix the following reagents in a 500 µl reaction tube and a total volume of 50 µl:

Concentration	Reagent
200 µM	dNTP
0.1-0.5 µM	Forward primer
0.1-0.5 µM	Reverse primer
5 µM	10x buffer (supplier)
20-200 pg/µl plasmid DNA **Or** 0.1-1 ng/µl cDNA library	Template DNA
2.5 U	*Pfu* polymerase*
Ad 50 µl	Distilled H_2O

*Depending on the recommendations of the manufacturer. *Pfu* can also be added after the initial denaturation step

3.1.2 Use a heated lid if available. Alternatively, overlay the sample with mineral oil.

3.1.3 Start temperature cycling:

Temperature	Time	Number of cycles	PCR step
94 °C	180 s	1	Initial denaturation
94 °C	30 s	15-20 times for plasmid DNA or 30-40 times for cDNA library	Denaturation
55-65 °C	30-60 s		Annealing
72 °C	30-240 s		DNA synthesis
60-72 °C	300 s	1	Terminal extension
4 °C	∞	1	Storage

3.1.4 Quantify the PCR fragment by comparing the band intensity with a DNA standard. Apply two different amounts of PCR product in separate lanes to find a band of equal intensity in the DNA standard, which has to be applied to the same gel. Alternatively, quantify via Spectrophotometer measurement.

3.1.5 Isolate the PCR product. If multiple bands are visible after gel electrophoresis, only isolate the PCR product of the expected size from the gel. Use of a DNA purification kit to extract the PCR fragment is recommended.

3.2 Donor vector generation

- To avoid evaporation and condensation in small reaction volumes, we recommend using 200 µl reaction tubes or incubation in 30 °C incubators.
- When working with *E. coli* cells it is important that they are not vortexed, as shearing forces can damage the cells.

3.2.1 Mix the following reagents into a new reaction tube for insertion of the GOI into the entry vector:

Amount	Reagent
7.5 µl	pENTRY-IBA51 (5 ng, dilute in distilled water)
12.5 µl	PCR product (2 nM, from section 3.1)
1 µl	12.5 mM ATP
1 µl	T4 DNA Ligase (1 U/µl)
1 µl	*Lgu*I restriction enzyme
2.5 µl	Buffer supplied with *Lgu*I

3.2.2 Close the reaction vessel thoroughly to avoid evaporation. Mix gently and incubate at 30 °C for 1 h.

3.2.3 Thaw a vial of competent *E. coli* cells on ice.

3.2.4 After incubation, pipet off an aliquot of 10 µl from the reaction mixture from step 1 and add it to the thawed competent *E. coli* cells.

3.2.5 Mix gently and incubate for 30 min on ice.

3.2.6 Mix gently and incubate for 5 min at 37 °C.

3.2.7 Mix gently and incubate for 2-5 min on ice.

3.2.8 Add 900 µl LB medium and shake for 45 min at 37 °C. This incubation step is necessary to express kanamycin resistance prior to platin on kanamycin for selection

3.2.9 Plate 100 µl on LB agar containing 50 mg/l kanamycin and 50 mg/l X-gal.

3.2.10 Centrifuge the residual 900 µl cell mixture for 30 sec in a microfuge, resuspend the cell sediment with 100 µl LB medium and plate the whole amount on a separate plate LB/kan/X-gal

3.2.11 Incubate plates over night at 37 °C (upside down).

3.3 Donor vector identification

3.3.1 Pick 5 white colonies and perform DNA mini preparation.

3.3.2 Perform analytical XbaI/HindIII restriction. A fragment with the length of the PCR product from 3.1 plus 40 bases is expected. Check your GOI for internal XbaI/HindIII restriction sites and consider the changed DNA fragment sizes resulting from additional restriction sites.

3.3.3 Select a putatively correct clone and confirm sequence via donor vector forward and reverse sequencing. Sequencing is recommended as PCR may lead to mutations and to improper product ends. The region flanking the GOI should have the sequence:

```
TCTAGAAAAGCGCGTCTCCAATG-GOI-GGGAGGAGACGCGCTAAAAGCTT
XbaI                                          HindIII
```

3.3.4 Dilute 1 µg of the verified donor vector plasmid DNA with water to a final concentration of 2 ng/µl and store at – 20 °C.

3.4 GOI transfer into the acceptor vector

3.4.1 Prepare a DTT/ATP Mix:
250 mM DTT
12.5 mM ATP
The mix can be stored in small aliquots at -20 °C

3.4.2 Mix the following reagents for insertion of the GOI into the chosen acceptor vector:

Amount	Reagent
7.5 µl	Accpetor vector (5 ng, dilute in distilled water)
2.5 µl	Buffer supplied with *Esp*3I
12.5 µl	PCR product (2 nM, from section 3.1)
1 µl	DTT/ATP Mix
1 µl	T4 DNA Ligase (1 U/µl)
0.5 µl	*Esp*3I (10 U/µl)

3.4.3 Close the reaction vessel thoroughly to avoid evaporation. Mix gently and incubate at 30 °C for 1 h.

3.4.4 Thaw a vial of competent *E. coli* cells on ice.

3.4.5 After incubation, pipet off an aliquot of 10 µl from the reaction mixture from step 2 and add it to the thawed competent *E. coli* cells.
Store residual reaction mixture (15 µl) at 2-8 °C for backup purposes.

3.4.6 Mix gently and incubate for 30 min on ice.

3.4.7 Mix gently and incubate for 5 min at 37 °C.

3.4.8 Mix gently and incubate for 2-5 min on ice.

3.4.9 Mix 10 µl of the mix with 90 µl LB medium. Plate the diluted mix and the remaining 100 µl on LB agar containing 100 mg/l ampicillin and 50 mg/l X-gal.

3.4.10 Incubate plates over night at 37 °C (upside down).

3.5 Destination vector identification

3.5.1 Pick 3 white colonies and perform DNA mini preparation.

3.5.2 pASG-IBA, pESG-IBA, pCSG-IBA and pDSG-IBA have *Xba*I/*Hind*III restriction sites that flank the expression cassette and may be used for confirmation of GOI integration. For exact calculation of expected restriction fragment length please refer to the appropriate acceptor vector data sheet.

3.5.3 Validate the correct sequence of your GOI by sequencing. Sequences for primers specific for the acceptor vector are listed in section 2.3.

3.5.4 Use the destination vector to transform the corresponding host.

4 ACCEPTOR VECTOR COLLECTION OVERVIEW

The following list provides information about the expression host, used promoter, available secretion signal and cloning site including N- or C-terminal tag as well as the Cat. No. of each expression vector. The vector name (e.g., pASG-IBA5) comprises the expression system (pASG = *E. coli*/tet) and the expression cassette (affinity-tag/position/secretion signal, IBA5 = *Strep*-tag®II/N-term/no secretion signal).

Host	*E.coli*	Mammalia			Name	Secretion	N-term	GOI	C-term	Resistance
Promoter	tet	CMV	CMV	CMV						
Vector	pASG	pDSG (MEXi)	pCSG	pESG						
Cat.No.	5-4000-001	5-5200-001	5-5000-001	5-4400-001	IBAwt1	no				Amp
	5-4005-001				IBA5	no				Amp
	5-4105-001	5-5222-001	5-5105-001	5-4505-001	IBA105	no				Amp
	5-4003-001				IBA3	no				Amp
	5-4103-001	5-5220-001	5-5103-001	5-4503-001	IBA103	no				Amp
	5-4045-001				IBA45	no				Amp
	5-4145-001				IBA145	no				Amp
	5-4043-001				IBA43	no				Amp
	5-4143-001				IBA143	no				Amp
	5-4001-001	5-5201-001	5-5001-001	5-4401-001	IBAwt2	yes				Amp
	5-4004-001				IBA4	yes				Amp
	5-4104-001	5-5221-001	5-5104-001	5-4504-001	IBA104	yes				Amp
	5-4002-001				IBA2	yes				Amp
	5-4102-001	5-5219-001	5-5102-001	5-4502-001	IBA102	yes				Amp
	5-4044-001				IBA44	yes				Amp
	5-4144-001				IBA144	yes				Amp

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