Biomek 2000 - LIC Insert Preparation Protocol

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Summary: This protocol describes the standard procedure for preparing LIC Insert Stocks on the Biomek 2000. Purified Target PCR DNA is normalized and 0.4 pmol DNA is digested in a 40 ul reaction with T4 DNA polymerase exonuclease digestion in the presence of dTTP yields LIC sticky ends

Materials/Reagents/Equipment	Vendor	Stock Number
Disposables		
PCR tubes = 96 well plate	Marsh, Perkin-Elmer	
Reagents		
T4 DNA polymerase	New England Biolabs	
Reaction Buffer: 1X T4 DNA Polymerase Buffer [50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 @ 25°C)]. Supplement with 50 µg/ml BSA and dNTPs* (not included in supplied 10X buffer). Incubate at temperature suggested for specific protocol.		
dTTP mixture, 100 mM, –20C	Pharmacia, Promega	
Water, double deionized, autoclaved, RT		
PCR Products, normalized to	BSGC	
Equipment		
Biomek 2000 Robot	Beckman	
PCR Machine 9600	Perkin-Elmer	
Pipetman		

Procedure
Setup PCR machine to run at 22 ^{o} C for 30 min, then 75^{o} C for 10 min, and terminate to 4 ^{o} C.
Normalize Target PCR products to 0.40 pmol per 20 ul (0.02 pmol/ul). Have PCR DNA in 96 well plate. [Plate type =]. Total volume of cleaned, normalized PCR insert should be at least 25 ul. [1 kb insert: 1 pmole = 0.6 ug]
Make Master Mix for T4 DNA Polymerase Treatment. Each reaction will require 4 ul of T4 Reaction Buffer, 1.0 ul of dTTP [100 mM], 1.0 ul of BSA [10 mg/ml], 13.5 ul water and then add 0.5 ul [2.5U] T4 DNA Pol. Use Table below to make Master Mix.
Dispense 20 ul Master Mix to each Target PCR. Most efficient dispensing would be with a 8 tip head, P200 tip that would allow mixing of 40 ul reaction. Dispensing from single tip might allow more efficient usage of Master Mix.
Transfer 20 ul of normalized PCR product to PCR reaction plate. Use same transfer tip to mix the reaction mix.
Incubate in PCR machine cycle above. Store –20 ^{o} C.
Usage: use 2.0 ul [0.02 pmol Insert DNA] for each LIC reaction.

Master Mix Table:

Component	1 Reaction	8 Reactions (x 8.8*)	+ 2 Reactions
10x T4 Buffer	4 ul	35.2 ul	8 ul
dTTP [100 mM],	1 ul	8.8 ul	2 ul
BSA [10 mg/ml]	1 ul	8.8 ul	2 ul
DTT [100 mM]	[2 ul]	17.6 ul	[4 ul]
Water,	13.5 ul [11.5 ul]	118.8 ul[101.2 ul	27 ul [23 ul]
T4 DNA Pol [5U/ul]	0.5 ul	4.4 ul	1 ul
			40 ul

* This would give 10% excess; 16 ul extra for 8 reactions. Question: would this be too little excess? For full plate of 8 x 12, this would be 192 ul excess. Would this be too much excess? Perhaps need to determine what is a good extra volume required for robot and add this as a constant amount regardless of the number of reactions.

Novagen protocol
0.2 pmol insert
1 unit T4 DNA pol (certified)
2.5 mM dNTP
5 mM DTT
2 ul Buffer 10x
water
Reaction volume 20 ul

22 C for 30 min, 75 C for 20 min

LIC Insert Preparation Datasheet
[note: replace this with Excel Datasheet]

Name		Date	

No.	Sample		