Things done from 2015-02-26T00:00:00-05:00 for this month report

Project: Genes involved in Tissue independent pol II recruitment

Background: What are the players which are involved in this process? So far we have Identified Lola and GAF through genomic approaches and functional studies.

Questions:

Question: Does Lola concentration actually go up during development? What about GAF? Looking at protein level.

Background: From the RNA-seq data we thick that Lola levels go up during development. But what about at protein level? What about GAF? Probably the protein and RNA levels will be the same.

Note: It looks like Lola concentration goes up during development and GAF also goes up but not that much compared to Lola

All Experiments:

Experiment: Western blot to analyze the changes in Lola concentrations during development

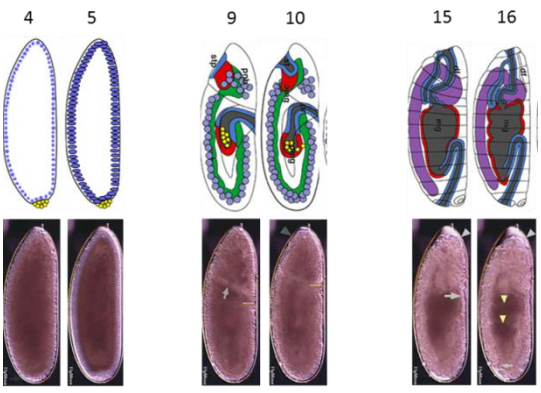
Description: We will sort particular embryonic stages and do western blot on them.

Mar 12: Western blot to analyze changes in Lola concentration.

Collect the embryos.

Dechorinate them with 50% bleech for 2mins.

Wash with distilled water.



Sorted EMBRYOS OF STAGES

4,5 ; 9,10; 15,16

Did not really separate the pre-mbt and post-mbt embryos. Had stages 4and5 together.

Sort uncrosslinked wild type ORER embryos for W.B about 35 embryos of the above stages.

Put about equal number of ORER embryos from each stage in the epi-tubes and keep them in ice.

Do all the steps on ice or 4c room as much as possible to reduce degradation.

Collecting embryos and prepare the sample for western blot
Collect the embryos. Dechorinate them with 50% bleech for 2mins. Wash with distilled water and sort them as said above.
Keep embryos in PBT before lysis.
Wash with A2 buffer before start. A2 buffer (25ml A2 buffer + 3P.I tablets) Add about 100ul A2, homogenize with RNA-prep pestle by hand by crushing them in cold room.
Further disrupt the membrane system by sonication using bioruptor. High power. For 5mins
Centrifuge at highest speed at 4°C for 10min, transfer the supernatant to new tubes.
While centrifuging the samples set up two gels. Label tubes for the supernatant and keep it in cold room. Prepare sample buffer (400ul lamelli buffer+40ul DTT). Set heat block to 95c

Western blot
Use Mini Protean TGX gel (4-15% grad) 10 well, 30ul, 5ul Precision Plus ProteinTM Dual Color Standards
Add 100ul of sample buffer to 80ul of sample supp. Heat at 95c for 5min.
Load the following amount of samples in the following order in the two gels.

Gel1:
1)Marker-5ul
2)15ul 4-5stg
3)15ul 4-5stg
4)15ul 4-5stg
5)25ul 4-5stg
6)25ul 9-10stg
7)25ul 15-16stg
8))Marker-5ul
9)
10)
Marker used: Broad range NEB

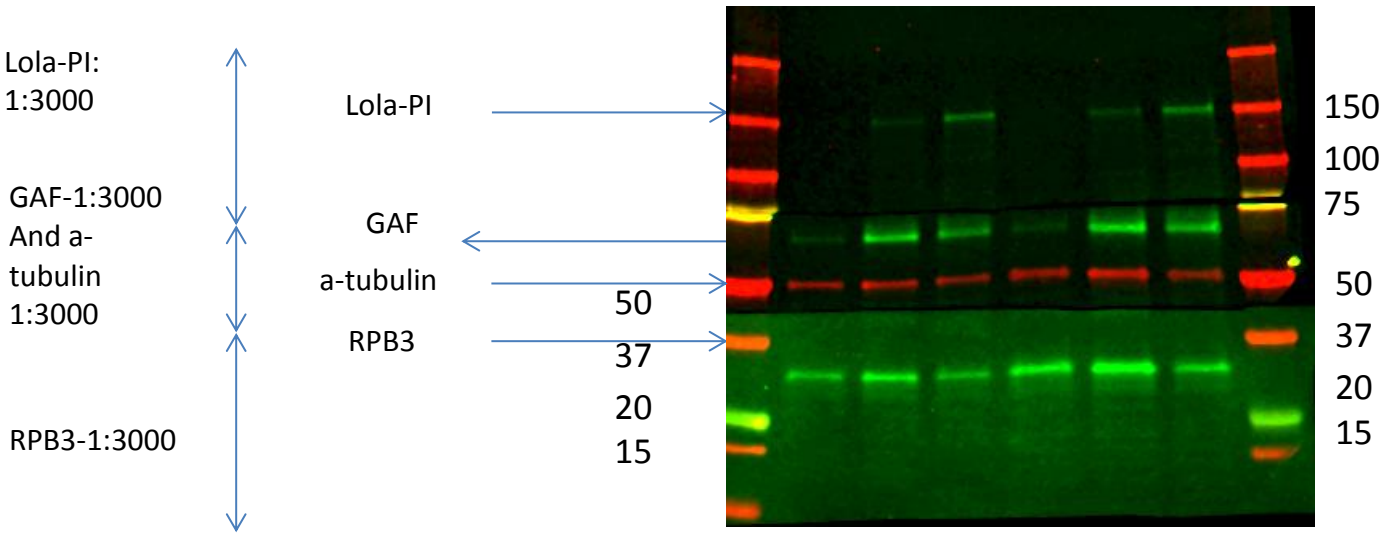
Lola-PI (1:3000)
GAF (1:3000)
RPB3 (1:3000)
a-tubulin(1:3000) sigma(T5168 from kausik’s lab)

Transfer the proteins to membrane by Iblot.

Dilute primary antibody in block buffer and incubate the membrane with primary antibody at RT o/n at 4c
The marked portions of each gel are incubated with respective antibodies.
Wash the membrane with TBS-T (1x TBS with 0.1% Tween 20) for 3 times, 5~10min each time (total 45mins)
Dilute the Secondary antibody in TBS-T 1:10000 T (1x TBS with 0.1% Tween 20). Incubate at RT for 45mins. Used mouse and rabbit fluorescent secondary antibodies from conaway lab.

Wash the membrane with TBS-T+0.1% tween for 3 times, 5~10min each time
Used LICOR instrument in the conaway lab for looking at the bands.

Results: A expected the levels of Lola go up during development. Might have to do pre-mbt and post mbt separately. Do not know why the levels of GAF are also going up, even though it peaks much earlier than Lola Will redo the experiment with different amounts of loading and separate pre and post MBT stages.



Mar 14: Western blot to analyze changes in Lola concentration.

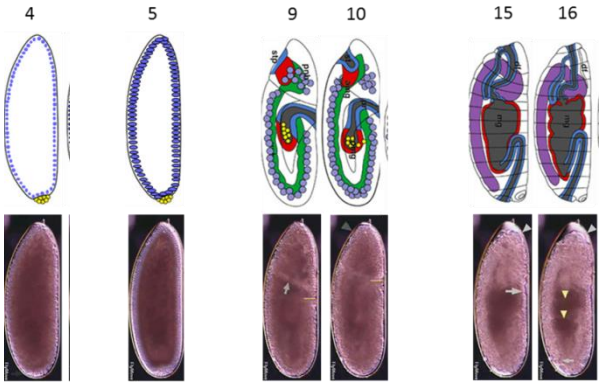
Note: This time will do prembt and mbt separately to see if GAF and Lola changes drastically at MZT.

Collect the embryos.

Dechorinate them with 50% bleech for 2mins.

Wash with distilled water.

Sorted EMBRYOS OF STAGES



4 and before(prembt); 5(Mbt) ; 9,10; 15,16

Sort uncrosslinked wild type ORER embryos for W.B about 35 embryos of the above stages.

Put about equal number of ORER embryos from each stage in the epi-tubes and keep them in ice.

Do all the steps on ice or 4c room as much as possible to reduce degradation.

Collecting embryos and prepare the sample for western blot
Collect the embryos. Dechorinate them with 50% bleech for 2mins. Wash with distilled water and sort them as said above.
Keep embryos in PBT before lysis.
Wash with A2 buffer before start. A2 buffer (25ml A2 buffer + 3P.I tablets) Add about 100ul A2, homogenize with RNA-prep pestle by hand by crushing them in cold room.
Further disrupt the membrane system by sonication using bioruptor. High power. For 5mins
Centrifuge at highest speed at 4°C for 10min, transfer the supernatant to new tubes.
While centrifuging the samples set up two gels. Label tubes for the supernatant and keep it in cold room. Prepare sample buffer (400ul lamelli buffer+40ul DTT). Set heat block to 95c

Western blot
Use Mini Protean TGX gel (4-15% grad) 10 well, 30ul, 5ul Precision Plus ProteinTM Dual Color Standards
Add 100ul of sample buffer to 80ul of sample supp. Heat at 95c for 5min.
Load the following amount of samples in the following order in the two gels.

Gel1:
1)Marker-5ul
2)12ul prembt stg
3)12ul mbt stg
4)12ul 9-10stg
5)12ul 15-16stg
6)25ul prembt stg
7)25ul mbt stg
8)25ul 9-10stg
9)25ul 15-16stg
10) Marker-5ul
Marker used: Broad range NEB

Lola-PI (1:3000)

GAF (1:3000)

RPB3 (1:3000)

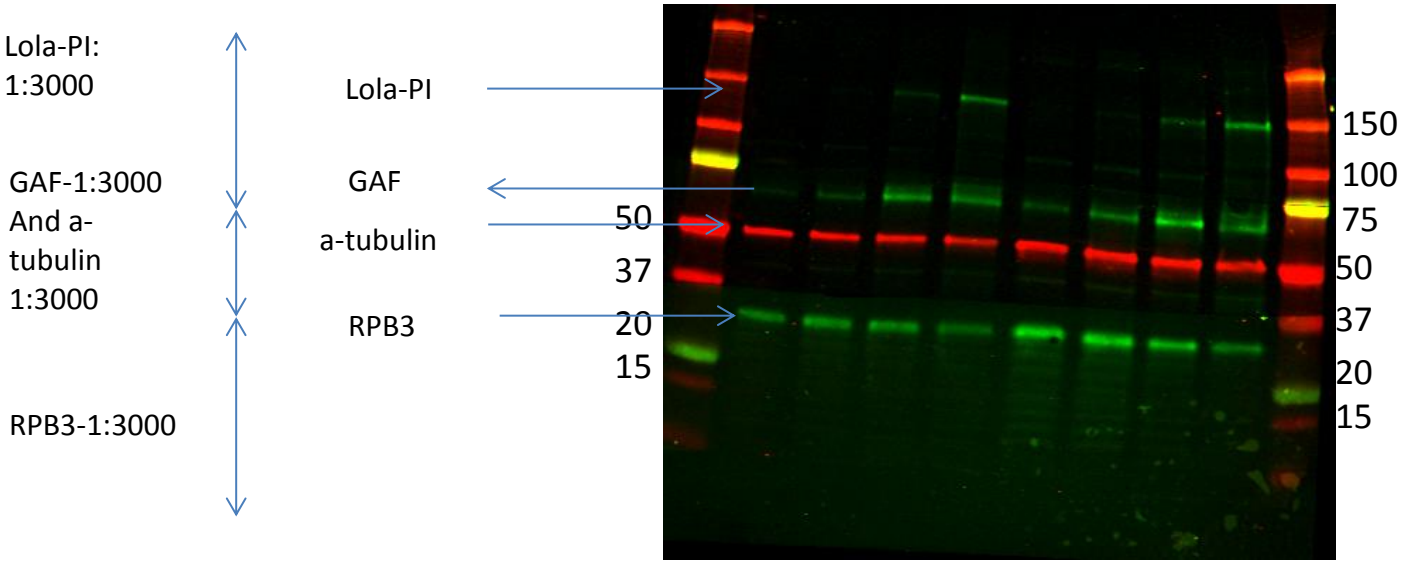
a-tubulin(1:3000) sigma(T5168 from kausik’s lab)

Transfer the proteins to membrane by Iblot.

Dilute primary antibody in block buffer and incubate the membrane with primary antibody at RT o/n at 4c
The marked portions of each gel are incubated with respective antibodies.
Wash the membrane with TBS-T (1x TBS with 0.1% Tween 20) for 3 times, 5~10min each time (total 45mins)
Dilute the Secondary antibody in TBS-T 1:10000 T (1x TBS with 0.1% Tween

20). Incubate at RT for 1 hr. Used mouse and rabbit fluorescent secondary antibodies from conaway lab.
Wash the membrane with TBS-T+0.1% tween for 3 times, total 45mins.
Used LICOR instrument in the conaway lab for looking at the bands.

Results: A expected the levels of Lola go up during development. This time did pre-MBT and MBT seperately. Do not know why the levels of GAF are also going up, even though it peaks much earlier than Lola.



Experiment: Immuno-staining to analyze the changes in Lola concentrations during development

Description: We will collect particular embryonic stages and do immunostaining on them.

Mar 12: Immunostaing to analyze changes in Lola concentration.

Notes:

For Immunostaining: Lola-PI

Control: DAPI, Lamin

Collect embryos of time points 0-2hrs, 2-4hrs, 4-8hrs, 14-17hrs

Dechorinate them with 50% bleech for 2mins.

Wash with distilled water.

Fix some of them with formaldehyde for immunostaining.

Embryos for immuno-staining
Collect the embryos
Add 50% bleach solution to apple plate for 1-2min then transfer the embryos to mesh basket and wash with tap water
Transfer embryos to 15ml tubes, add 140ul of formaldehyde, 2.3ml of crosslinking solution and 7.5ml of heptante. Fix for 30-35mins.
Centrifuge at 1900rpm for 1min and discard the supernatant
Add 5ml heptane and 5ml MeOH, shake by hand for 1-2min. **At this point, the embryos should sink to rapidly to the bottom of the vial, and there should be vitalin at the interface of the MeOH and heptane. If this doesn’t occur, incubate longer or shake more vigorously
Centrifuge 1900rpm 1min, discard the supernatant, wash with 10ml MeOH three times, and keep embryos in MeOH at -20°C.

Crosslinking buffer 50mM Hepes 1mM EDTA 0.5mM EGTA 100mM NaCl

	Immunostaining
1	Rehydrate embryos with MeOH/PBS gradient, 90%, 75%, 50%, 25% 5min each step, and then wash with Immuno-block 3 times
2	Incubate embryos with immuno-block at 4c o/n
3	Dilute the primary antibody in immuno-block 1:500, 1:750 of Lola-PI, lamin as control. Also tried snail as control but did not do imaging. As I am not sure what this will tell.
4	Incubate on a rocker at 4°C overnight.
5	Remove and save the antibody (could be used again)
6	Wash the embryos with immuno-block at RT 1x5min, then 2x30min on a rocker
7	Dilute the Alexafluor Dye in immuno-block, 1:1000, incubate at RT for 2h (the dye is light sensitive! Incubate at dark place or in a container)
8	Wash 3 times with PBT: 1st and 2nd wash: simply allow the embryos to settle before proceeding to the next wash 3rd wash: 30min on a rocher
5	Dilute DAPI in PBT, 1:1000, incubate the embryos for 15min, wash twice with PBT at RT, 5 min each time.
6	Take out all the liquid, add 100~200ul 2.5% Dabco to the embryo, wait until the embryos are set down, mount 60~80ul on each slide.
	Immuno-block 1x PBS

	0.1% Triton, 0.5% BSA
	2.5% Dabco 90% glycerol 2.5% Dabco

Results: A expected the levels of Lola go up during development. Always used cells in the surface to expose them the same. Talked with Brain about how to do it quantitatively. Will do it later. Will have to image more embryos per stage. Will do nuclear quantification with Brain and Jay. Even though can do this in a simpler way, this imaging with brain at different time point embryos will be an exercise for our reporter assay.

Is Lola involved in recruiting pol II to the late opening genes?

Background: So far we have found correlation between increase in Lola at 1417hrs compared to 24hrs with corresponding increase in GAF and pol II in the lola target genes. We think that lola plays a role in recruiting pol II to these genes.

All Experiments:

Experiment:

Rescue Lola mutants with Lola from Bac construct. The 38662 flies have Lola-PI tagged with GFP. While 38661 have Lola-PI without tag but Lola-PG tagged with GFP.

Description: These experiments will serve many purposes. First it will allow us to test whether the Lola mutant that we have and the phenotype of those mutants is because of mutations in Lola. They will serve as rescue experiments. Second it will help to test the GFP tagged version that we used for pull-down is function (The ChIP on the tagged protein look similar to endogenous untagged)

The 38662 flies have Lola-PI tagged with GFP. While 38661 have Lola-PI without tag but Lola-PG tagged with GFP.

Cross scheme for the Lola-PI-GFP flies for rescue experiment (38662):

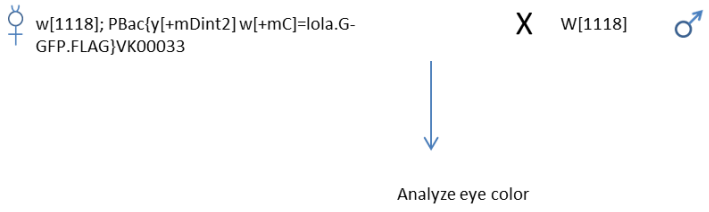
Cross scheme for the Lola-PG-GFP flies for rescue experiment (38661)::

Have so far done the first cross and collecting the virgins from that cross and going to do the second cross. But I am not sure if I will have enough flies. Also the entire scheme depends on the ability to differentiate between CyoROI and Cyo. But in the w[1118]; +/ CyO,amos[Roi]; PBac{y[+mDint2] w[+mC]=lola.G-GFP.FLAG}VK00033 / TM3,Sb,Ser flies I am not sure I can see the ROI. Going to ask Cindi.

Also have crossed the 38662 flies with W1118 flies to see if we can differentiate between the homozygous PBac{y[+mDint2] w[+mC]=lola.G-GFP.FLAG}VK00033 vs heterozygous based on the intensity of eye color. It looks like we can the heterozygous is lighter than the parent.

Cross the 38662 flies with W1118 flies:

Cross scheme:



Experiment: Rescue Lola mutants with Lola cDNA with or without BTB domain.

Description: These experiments will serve many purposes. First it will allow us to test whether the Lola mutant that we have and the phenotype of those mutants is because of mutations in Lola. They will serve as rescue experiments. Second it will help to test if the BTB domain, presumably its interactions with GAF required for Lola function. We can also use these constructs to overexpress Lola at early embryogenesis and test if the timing of pol II recruitment could be shifted. We can also use the overexpression system for pulldown and massspec experiments.

The cloning strategy to do the overexpression:

Will do overexpression from different promoters.

Act5c, UAS and Hsp70

Cloning of Lola-PI cDNA with 3X-Flag tag and with or without BTB domain in to pUASP-attb vector

Resources:

1) Synthesized Lola-PI 5’end cDNA in vector and in a PCR fragment(from 12/1/2014).(sequence verified)
2) Lola-PI 3’end cDNA in vector from Molbio(LD17006).(sequence verified)
3) pUASP-attb vector (sequence verified), pAW, pHW vectors as source for Act5c promoter and Hsp70 promoter. Got pAW, pHW vectors from Gibson lab.

Plan:

1) Use GIBSON assembly to put together all the pieces in the pUASP-attb vector. Design the needed primers.

The orientation of the Lola3’ fragment in the LD17006 clone might be different than initially planned. Also have to use GIBSON assembly to easily create the deletion without the BTB domain. So I am planning to use GIBSON assembly for all.

Sequence of the lola-PI cDNA:

BTB domain

/translation="MDDDQQFCLRWNNHQSTLISVFDTLLENETLVDCTLAAEGKFLK

AHKVVLSACSPYFATLLQEQYDKHPIFILKDVKYQELRAMMDYMYRGEVNISQDQLAA

LLKAAESLQIKGLSDNRTGGGVAPKPESSGHHRGGKLSGAYTLEQTKRARLATGGAMD

TSGDVSGSREGSSSPSRRRRKVRRRSMENDAHDNSNSSVLQAAASNQSILQQTGAGLA

VSALVTTQLSSGPAAGTSSQASSTQQQQPLTSTNVTKKTESAKLTSSTAAPASGASAS

AAVQQAHLHQQQAQTTSDAINTENVQAQSQGGAQGVQGDDEDIDEGSAVGGPNSATGP

NPASASASAVHAGVVVKQLASVVDKSSSNHKHKIKDNSVSSVGSEMVIEPKAEYDDDA

HDENVEDLTLDEEDMTMEELDQTAGTSQGGEGSSQTYATWQHDRSQDELGLMAQDAQQ

RDPQASKQDKGEQTEGAQDEFELDDCLLESNDIVITQNKDGFVLHVKKLGNITAAKLE

ENQAVAQQQGQAAVTVTGPAGQPTPTITELLNAAAASHSEPKPTLTTLTSTPIKLPSS

ECELINIKKIIPATTTIATHHPHTSSTIIHPHHIIQHVSQEPHHQEHHQQHQTIHIEE

VPQTSQQHHQQQHHHQLQTVQPTHTQVQSIITAHPGQTINLVGLRNVQLADSKPIASR

IRYSRGKIIGPTVQNLQIVETHEPIQHQHHELSDGTKYEISEIDLNNPNASAAIISDL

VKYAEIDDIELPDGTKIGIGFAPSEITEHMQTSGGETHITTIEHEPQELQTVHQHEQT

QQTHHIHAGQLQTHHIQTVVQSSSGQQQHDQQQHHQHHSIELQDDDGVETITPEELGM

HDSSKSYTILTTRPMKEESEHDPSGMTYELSLSDSSLGPCDDPESRYVCRHCGKKYRW

KSTLRRHENVECGGKEPCHPCPYCSYKAKQRGNLGVHVRKHHPEKPQLESKRGRKV"

Lola-PI sequence:

(Highlighting the Coding region)
Start and Stop codon

BTB domain

GTTTCATTCGGTTTCGTGCGCTCGGGGATTTTGTTTGGTTCTCTGCTCTGTGCGGCAGATCGCGAGGCAA
CGCGTGCGGTTTGCTGGCTAAATTCTACATATACACATTCAACATCCGACGGCCGAAGCGGAGCTAGAAT
AGGAAAACCAACCTTTGCAGTGCTAATTATTTACAACACAACACATAACTGCTGACACACTAAACAAAAC
TGAAAAAAGAACCAAGGGCGAAAGCTGTTTATTTTGTTTAGGCACCTAAGTTTGGCCAAATTATAATCAA
AAAACGAGTGGCGAAAAAAACAGCAAGTCAGAGAGAGCGCTCGACGTTCGGCCCCAAGGTCGTGGGCCCA
AACCCCCAGGATCCATTTCCAACAACCCCATCCAACCACCCGAGAAACCAGAATGGATGACGATCAGCAG
TTTTGTTTGCGGTGGAACAACCACCAGAGCACACTGATCAGCGTGTTCGACACGTTGCTGGAGAACGAGA
CTCTAGTCGATTGCACGCTAGCCGCCGAGGGCAAATTTCTCAAGGCCCACAAGGTGGTGCTGTCAGCATG
CAGTCCCTACTTTGCTACCTTACTACAAGAACAGTACGACAAACATCCCATCTTTATACTCAAGGATGTC
AAGTACCAAGAGCTGCGCGCCATGATGGACTACATGTACCGCGGCGAGGTCAATATCTCGCAGGATCAGC
TGGCCGCTCTGCTCAAGGCCGCCGAATCGCTTCAGATCAAGGGCCTTTCGGACAATCGCACTGGCGGCGG
AGTAGCTCCCAAGCCAGAGTCCTCCGGCCATCATCGCGGCGGTAAGCTGAGCGGTGCCTACACACTGGAG
CAAACTAAGCGGGCTCGACTGGCCACCGGCGGAGCGATGGATACGTCTGGCGATGTGTCCGGTTCGCGCG

AGGGCTCCTCGAGTCCGTCGCGTCGTCGCCGAAAAGTCCGACGTCGCAGCATGGAGAATGATGCCCACGA
CAACTCAAACTCGTCCGTGTTACAAGCCGCCGCCTCGAATCAGTCAATCCTCCAGCAGACAGGGGCCGGT
TTGGCCGTCTCCGCTTTGGTCACCACCCAGTTGTCCAGCGGACCGGCAGCCGGAACCAGCAGCCAAGCGT
CGTCGACCCAACAACAGCAGCCATTGACCAGCACCAACGTTACCAAAAAGACTGAAAGCGCTAAACTAAC
ATCCTCGACAGCCGCCCCAGCGAGCGGAGCATCTGCGTCAGCGGCTGTACAACAGGCCCATCTGCATCAG
CAGCAGGCGCAGACCACAAGCGATGCCATTAACACCGAGAATGTACAAGCCCAGAGCCAAGGTGGCGCCC
AAGGCGTCCAAGGCGATGACGAGGACATTGATGAGGGTAGTGCCGTTGGCGGACCAAACTCGGCCACCGG
ACCCAATCCCGCCTCCGCCTCTGCATCCGCCGTCCATGCCGGAGTTGTGGTAAAGCAGCTGGCCAGCGTT
GTGGACAAATCGTCGTCGAATCACAAACATAAGATCAAAGACAACAGCGTGTCATCAGTGGGCTCCGAAA
TGGTTATTGAACCCAAAGCCGAATACGATGACGATGCGCACGATGAGAATGTTGAGGATTTGACACTGGA
CGAGGAGGACATGACAATGGAGGAGCTGGACCAGACGGCCGGCACCAGCCAGGGTGGCGAAGGATCTAGT
CAAACATATGCAACATGGCAGCACGACAGATCTCAGGATGAACTTGGACTAATGGCACAGGATGCACAGC
AACGGGATCCCCAAGCATCCAAGCAGGACAAGGGCGAGCAGACCGAAGGAGCACAGGATGAATTCGAACT
GGACGACTGTTTGCTGGAGAGCAACGACATAGTCATCACCCAGAACAAGGATGGATTCGTGTTGCACGTA
AAGAAGCTGGGTAACATTACGGCTGCCAAGCTGGAGGAGAACCAGGCGGTGGCACAACAACAGGGCCAAG
CGGCAGTGACTGTCACCGGGCCGGCTGGACAACCCACGCCCACAATCACGGAACTCTTGAATGCCGCCGC
CGCTTCGCACTCGGAACCCAAACCCACACTGACCACTCTGACCAGTACGCCCATTAAATTGCCGTCATCG
GAATGCGAACTTATCAACATTAAAAAGATTATACCGGCAACCACAACAATTGCCACACATCATCCGCACA
CGAGTTCGACAATCATACATCCCCATCACATCATTCAGCATGTGTCCCAGGAACCGCACCACCAGGAGCA
CCATCAGCAGCATCAGACTATTCACATTGAGGAGGTGCCGCAGACTTCGCAACAGCACCACCAGCAGCAG
CATCACCACCAGCTTCAGACGGTCCAACCGACCCACACCCAAGTACAAAGCATAATCACAGCTCATCCGG
GCCAGACTATAAACCTGGTGGGTCTGCGCAATGTGCAGTTGGCCGATTCGAAGCCCATAGCCTCGAGGAT
ACGATATTCTCGTGGAAAGATCATCGGGCCGACGGTACAAAACCTGCAGATCGTGGAAACCCATGAGCCC
ATCCAGCATCAACATCACGAGCTGTCGGATGGCACCAAGTATGAGATTAGCGAGATTGATCTGAACAATC
CCAATGCATCGGCGGCAATCATAAGTGACTTAGTGAAGTATGCCGAGATCGATGATATCGAGCTGCCCGA
CGGCACTAAGATTGGCATCGGCTTTGCGCCCTCGGAGATTACTGAGCATATGCAGACCTCCGGCGGAGAG
ACGCACATCACTACAATTGAGCACGAGCCGCAGGAACTGCAAACGGTCCATCAGCACGAACAGACGCAGC
AGACGCATCACATACATGCCGGCCAACTGCAGACGCATCATATCCAGACCGTGGTGCAGTCCAGCAGTGG
CCAGCAGCAGCACGATCAGCAGCAGCATCATCAGCACCATAGCATTGAGCTGCAGGACGACGATGGTGTG
GAGACAATCACACCCGAAGAGCTTGGTATGCACGATTCGAGCAAGAGCTACACCATTCTCACCACTCGTC
CCATGAAGGAGGAGTCGGAGCACGATCCTTCGGGCATGACCTACGAACTGTCGCTGAGCGACTCGTCTCT
GGGTCCATGCGATGATCCAGAGTCGCGGTATGTGTGCCGCCATTGCGGAAAGAAATATCGCTGGAAGTCG
ACGCTGCGTCGCCACGAGAACGTCGAGTGTGGTGGCAAGGAGCCGTGTCATCCGTGCCCGTACTGCAGCT
ACAAGGCCAAGCAGCGGGGTAATCTCGGAGTGCATGTGCGCAAACATCATCCGGAGAAGCCGCAGCTAGA
GAGCAAGCGAGGCCGCAAGGTCTAGGCGAAAGTTGGGAGGATTGTGAGTACACAACTGTTGGGTGTAAAG
AAGTAACCATTTTTGAGGTTAAGACCAGTTTCATTATTATTACAACTATTTTGTATCATTTTATAAACGT
GTACACCCTATTAAGCTTAAAGTTCTTATTTGTTGATTATTTTTATTAAGTTTATAAGTTTTGACTTCCA
CTCGGTTCTCAAAGGTTCAATTTTTCAAACAAACAAGCATTGTGCGCACTCAATTACATTTTTATCAGGC
TTTAACTTTAATGTCTTCATATTTAGTAGAATTGGTTATGTATGTCCTTTAATTCATCGTAATTTTCAAA
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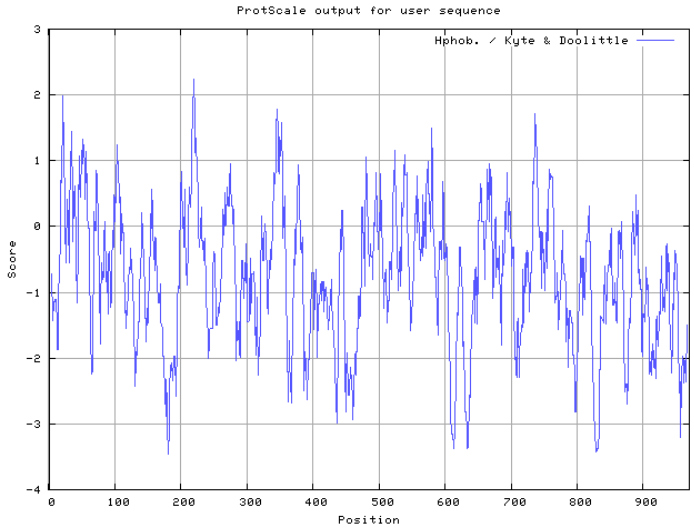
GAAATAACACAAAAAAAATATAGGATCAACTATATTAATGGAAAATAGATTAACATTAAGAATAATTAAA
AACTTATTTCTGTATGTATGTGTGTGTGCTCTCAAAAAAATATGAAAATATATATAAAAAATCTTGATTC
AAATCTAAGTTATGTAGAAAAATCTTAACTGAAGTCCTGAAATTTTTTTAATTTTTTACTGCAATTCAAC
GTTGGTGCACATAAATTTCATGTAACCTATTTAAACAAAACCTATCGACATACAAAAATTGAAGAAAATT
GTAAACTTATCTATATTGGAGGCTTGCAAAAATAATTTTGTAAGAATGCGTTTTCAAATCTTTATATATA
TATGATATATGAATAGCATTCAAAAAATATGAGTGTAAGGAAGTTATTTTTCTGTAAATTGCAAAAACTT
AACAATCTACTCGAACTTAGTAAAATTGATTGTACATCGGAATAGCTGAGTAAAGTGTGAACATTTTGAA
ACATATTTCCGCAGAAAAAATAAAATATAACAAAAATCT

Hydrophobicity plot for Lola-PI:

Looking at the Hydrophobicity plot for the Lola-PI to decide if there would be a problem when we remove the BTB domain in the mutant. There is not much clear idea of what I am looking for in this plot. if there for some reason the high hydrophobic region behind the BTB domain, now it might get exposed to water in the mutant. But I do not find any strong hydrophobic region in the protein.



Would the protein folding be affected by bringing together to non-native sequences in the deletion mutant? We don’t know but may be not because of the following reasons:

1) These sequences on either side might fold independently because there is the BTB domain in the native sequence in between them.
2) There is also a stretch of three glycine and some proline after the BTB domain.

Sequences of some of the fragments to be used in the GIBSON assembly to make the lola-cDNA:

5’end without the BTB:

GTTTCATTCGGTTTCGTGCGCTCGGGGATTTTGTTTGGTTCTCTGCTCTGTGCGGCAGATCGCGAGGCAACGCGTGCGGTTTGCTG
GCTAAATTCTACATATACACATTCAACATCCGACGGCCGAAGCGGAGCTAGAATAGGAAAACCAACCTTTGCAGTGCTAATTATTT
ACAACACAACACATAACTGCTGACACACTAAACAAAACTGAAAAAAGAACCAAGGGCGAAAGCTGTTTATTTTGTTTAGGCACCT
AAGTTTGGCCAAATTATAATCAAAAAACGAGTGGCGAAAAAAACAGCAAGTCAGAGAGAGCGCTCGACGTTCGGCCCCAAGGTC
GTGGGCCCAAACCCCCAGGATCCATTTCCAACAACCCCATCCAACCACCCGAGAAACCAGAATGGATGACGATCAGCAGTTTTGTT
TGCGGTGGAACAACCACCAGAGCACACTGAT

3’end fragment with 3x Flag:

CCAATGCATCGGCGGCAATCATAAGTGACTTAGTGAAGTATGCCGAGATCGATGATATCGAGCTGCCCGA
CGGCACTAAGATTGGCATCGGCTTTGCGCCCTCGGAGATTACTGAGCATATGCAGACCTCCGGCGGAGAG
ACGCACATCACTACAATTGAGCACGAGCCGCAGGAACTGCAAACGGTCCATCAGCACGAACAGACGCAGC
AGACGCATCACATACATGCCGGCCAACTGCAGACGCATCATATCCAGACCGTGGTGCAGTCCAGCAGTGG
CCAGCAGCAGCACGATCAGCAGCAGCATCATCAGCACCATAGCATTGAGCTGCAGGACGACGATGGTGTG
GAGACAATCACACCCGAAGAGCTTGGTATGCACGATTCGAGCAAGAGCTACACCATTCTCACCACTCGTC
CCATGAAGGAGGAGTCGGAGCACGATCCTTCGGGCATGACCTACGAACTGTCGCTGAGCGACTCGTCTCT

GGGTCCATGCGATGATCCAGAGTCGCGGTATGTGTGCCGCCATTGCGGAAAGAAATATCGCTGGAAGTCG
ACGCTGCGTCGCCACGAGAACGTCGAGTGTGGTGGCAAGGAGCCGTGTCATCCGTGCCCGTACTGCAGCT
ACAAGGCCAAGCAGCGGGGTAATCTCGGAGTGCATGTGCGCAAACATCATCCGGAGAAGCCGCAGCTAGA
GAGCAAGCGAGGCCGCAAGGTCGGAGGA GAC TAC AAG GAC CAC GAC GGT GAC TAC AAG GAC CAC
GAC ATC GAC TAC AAG GAC GAC GAC GAC AAG TGA TAG

GGAGGA – linker sequence

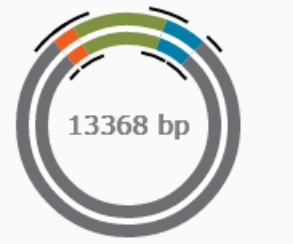
GAC TAC AAG GAC CAC GAC GGT GAC TAC AAG GAC CAC GAC ATC GAC TAC AAG GAC GAC GAC GAC AAG TGA - 3x flag

TAG – stop codon

Other details can be found from the assembled sequence.

Cloning without the BTB domain and with 3x-Flag tag:

UAS promoter:



User Selected Settings

Product Version	E2611 - Gibson Assembly Master Mix
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	30 bp
Min. Fragment Length	150 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement

- PUASP-attb
- 5'_and_first_21aminoacids
- Fragment_from_synthesized_5'end

- 3’end_fragment_with_3x_Flag
- PUASP-attb

Required Primers

---	CAGATCCACTAGTGGCCTATG	PUASP-attb	Rev	62.3°C	64.6°C	view
PUASP-attb	ccgcggccgcataggccactagtggatctgGTTTCATTCGGTTTCGTG	5'_and_first_21aminoacids	Fwd	56.4°C	59.4°C	view
Fragment_from_synthesized_5'end	cgccgccagtgcgattGATCAGTGTGCTCTGGTG	5'_and_first_21aminoacids	Rev	60.5°C	59.4°C	view
5'_and_first_21aminoacids	cagagcacactgatcAATCGCACTGGCGGCGGA	Fragment_from_synthesized_5'end	Fwd	71.5°C	70.9°C	view
3’end_fragment_with_3x_Flag	gccgccgatgcattggGATTGTTCAGATCAATCTCGCTAATCTCATACTTGG	Fragment_from_synthesized_5'end	Rev	67.9°C	70.9°C	view
Fragment_from_synthesized_5'end	ttgatctgaacaatcCCAATGCATCGGCGGCAA	3’end_fragment_with_3x_Flag	Fwd	67.6°C	66.0°C	view
PUASP-attb	taacgttaacgttcgaggtcgactctagagCTATCACTTGTCGTCGTCGTC	3’end_fragment_with_3x_Flag	Rev	63.0°C	66.0°C	view
---	CTCTAGAGTCGACCTCGAAC	PUASP-attb	Fwd	61.6°C	64.6°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are

assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.

Assembled Sequence

GCTGCACCTGCAAAAGGTCAGACATTTAAAAGGAGGCGACTCAACGCAGATGCCGTACCTAGTAAAGTGATAGAGCCTGAACCAGAAAAGA
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HiFi DNA Assembly® Protocol

Optimal Quantities
NEB recommends a total of 0.03–0.2 pmols of DNA fragments when 1 or 2 fragments are being assembled into a vector and 0.2–0.5 pmoles of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

50 ng of 5000 bp dsDNA is about 0.015 pmols.

50 ng of 500 bp dsDNA is about 0.15 pmols.

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Assembly Protocol

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly*	4-6 Fragment Assembly**	Positive Control†
Recommended DNA Ratio	vector:insert = 1:2	vector:insert = 1:1	
Total Amount of Fragments	0.03–0.2 pmols* X µl	0.2–0.5 pmols* X µl	10 µl
Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

2. * Optimized cloning efficiency is 50–100 ng of vectors with 2 fold excess of inserts. Use 5 times more inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.
   ** To achieve optimal assembly efficiency, it is recommended to design ≥ 20 bp overlap regions between each fragment with equimolarity (suggested: 0.05 pmol each).
   † Control reagents are provided for 5 experiments.
   ‡ If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional Assembly Master Mix.
3. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

   Note: Reaction times less than 15 minutes are generally not recommended. Extended incubation times (up to 4 hours) have been shown to improve assembly efficiencies in some cases. Do not incubate the assembly reaction overnight.

4. Transform NEB 5-alpha Competent E. *coli* cells (provided in the cloning kit or purchased separately from NEB) with 2 µl of the assembled product, following the appropriate transformation protocol.

Transformation Protocols

Transformation with chemically competent cells.

1. Thaw chemically competent cells on ice.
2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube (if necessary).
3. If the chemically competent cells are from New England Biolabs, add 2 µl of assembled product to NEB competent cells and go to step 4 directly. If competent cells are purchased from other manufacture, dilute assembled products 4-fold with H_2O prior transformation. This can be achieved by mixing 5 µl of assembled products with 15 µl of H_2O. Add 2 µl of the diluted assembled product to competent cells.
4. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds.* Do not mix.
6. Transfer tubes on ice for 2 minutes.
7. Add 950 µl of room temperature SOC media* to tubes.
8. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
9. Warm selection plates to 37°C.
10. Spread 100 µl of the cells onto the plates with appropriate antibiotics. Use Amp plates for positive control sample.
11. Incubate plates overnight at 37°C.

    * Please note: Follow the manufacturer's protocols for the duration and temperature of the heat shock step, as well as the optimal medium for recovery. Typically, transformation of our positive control assembly product will yield more than 100 colonies on an Amp plate with greater than 80% colonies containing inserts.

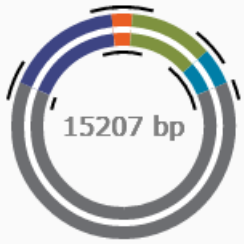
    NEB recommends NEB 5-alpha Competent *E. coli* (NEB #C2987) for transformation of Gibson Assembly products. It is also possible to use other NEB competent *E. coli* strains, with the exception of BL21, BL21(DE3), Lemo21(DE3) and Nico21(DE3). For example, Shuffle T7 Express Competent *E. coli* can be used for the expression of a difficult to express protein. When using competent *E. coli* from a vendor other than NEB, we have seen decreased robustness of transformation with the Gibson Assembly reaction.

Transformation with electrocompetent cells.

1. Thaw electrocompetent cells on ice.
2. Transfer 50 µl of electrocompetent cells to a pre-chilled electroporation cuvette with 1 mM gap.
3. Dilute assembled products 3-fold with H_2O prior electroporation. This can be achieved by mixing 5 µl of assembled products with 10 µl of H_2O. Add 1 µl of the diluted assembly product to electrocompetent cells.
4. Mix gently by pipetting up.

5. Once DNA is added to the cells, electroporation can be carried out immediately. It is not necessary to incubate DNA with cells.
6. Add 950 µl of room temperature SOC media to the cuvette immediately after electroporation.
7. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the plates.
10. Incubate overnight at 37°C.

Act5c promoter:



User Selected Settings

Product Version	E2611 - Gibson Assembly Master Mix
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	30 bp
Min. Fragment Length	150 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement

- PUASP-attb
- Actin_5c_promoter
- 5'_and_first_21aminoacids
- Fragment_from_synthesized_5'end
- 3’end_fragment_with_3x_Flag
- PUASP-attb

Required Primers

---	CTAGTATGTATGTAAGTTAATAAAACCC	PUASP-attb	Rev	56.9 °C	59.9 °C	view
PUASP-attb	gggttttattaacttacatacatactagGCATGCAATTCTATATTCTAAAAACACAAATG	Actin_5c_promoter	Fwd	modified	modified	view
5'_and_first_21aminoacids	gaaaccgaatgaaacATCTGGATCCGGGGTCTC	Actin_5c_promoter	Rev	63.8 °C	64.7 °C	view
Actin_5c_promoter	accccggatccagatGTTTCATTCGGTTTCGTG	5'_and_first_21aminoacids	Fwd	56.4 °C	59.4 °C	view
Fragment_from_synthesized_5'end	cgccgccagtgcgattGATCAGTGTGCTCTGGTG	5'_and_first_21aminoacids	Rev	60.5 °C	59.4 °C	view
5'_and_first_21aminoacids	cagagcacactgatcAATCGCACTGGCGGCGGA	Fragment_from_synthesized_5'end	Fwd	71.5 °C	70.9 °C	view
3’end_fragment_with_3x_Flag	gccgccgatgcattggGATTGTTCAGATCAATCTCGCTAATCTCATACTTGG	Fragment_from_synthesized_5'end	Rev	67.9 °C	70.9 °C	view
Fragment_from_synthesized_5'end	ttgatctgaacaatcCCAATGCATCGGCGGCAA	3’end_fragment_with_3x_Flag	Fwd	67.6 °C	66.0 °C	view
PUASP-attb	taacgttaacgttcgaggtcgactctagagCTATCACTTGTCGTCGTCGTC	3’end_fragment_with_3x_Flag	Rev	63.0 °C	66.0 °C	view
---	CTCTAGAGTCGACCTCGAAC	PUASP-attb	Fw	61.6 °C	59.9 °C	view

			d			

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.

Assembled Sequence

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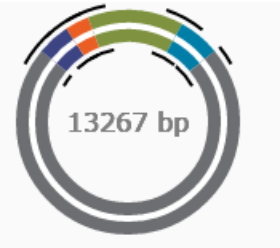
Hsp70 promoter:

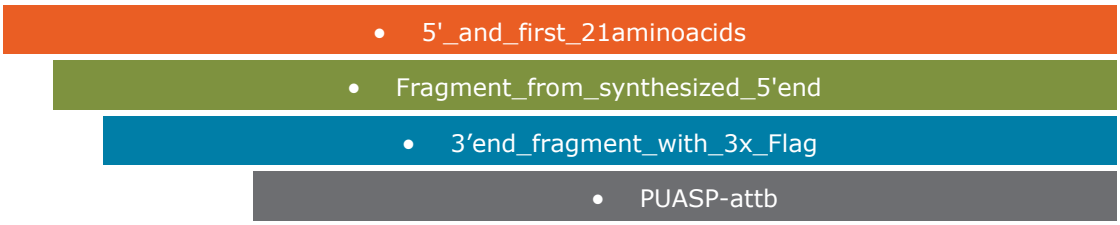


User Selected Settings

Product Version	E2611 - Gibson Assembly Master Mix
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	30 bp
Min. Fragment Length	150 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement

- PUASP-attb
- Hsp70_promoter



Required Primers

---	CTAGTATGTATGTAAGTTAATAAAACCC	PUASP-attb	Rev	56.9°C	59.9°C	view
PUASP-attb	atgggttttattaacttacatacatactagCATGCTGGAGCTATCCGC	Hsp70_promoter	Fwd	63.9°C	64.1°C	view
5'_and_first_21aminoacids	gaaaccgaatgaaacTTTGCTCAGCTTGCTTCG	Hsp70_promoter	Rev	61.1°C	64.1°C	view
Hsp70_promoter	agcaagctgagcaaaGTTTCATTCGGTTTCGTG	5'_and_first_21aminoacids	Fwd	56.4°C	59.4°C	view
Fragment_from_synthesized_5'end	cgccgccagtgcgattGATCAGTGTGCTCTGGTG	5'_and_first_21aminoacids	Rev	60.5°C	59.4°C	view
5'_and_first_21aminoacids	cagagcacactgatcAATCGCACTGGCGGCGGA	Fragment_from_synthesized_5'end	Fwd	71.5°C	70.9°C	view
3’end_fragment_with_3x_Flag	gccgccgatgcattggGATTGTTCAGATCAATCTCGCTAATCTCATACTTGG	Fragment_from_synthesized_5'end	Rev	67.9°C	70.9°C	view
Fragment_from_synthesized_5'end	ttgatctgaacaatcCCAATGCATCGGCGGCAA	3’end_fragment_with_3x_Flag	Fwd	67.6°C	66.0°C	view
PUASP-attb	taacgttaacgttcgaggtcgactctagagCTATCACTTGTCGTCGTCGTC	3’end_fragment_with_3x_Flag	Rev	63.0°C	66.0°C	view

---	CTCTAGAGTCGACCTCGAAC	PUASP-attb	Fwd	61.6°C	59.9°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.

Assembled Sequence

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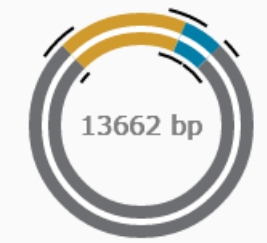
Cloning with the BTB domain and with 3x-Flag tag:

UAS promoter:



User Selected Settings

Product Version	E2611 - Gibson Assembly Master Mix
No. of Fragments	2-3 fragments (including vector)
Min. Overlap	30 bp
Min. Fragment Length	150 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement

- pUASP-attb
- 5'_fragment
- 3'_fragment_with_3x_flag
- pUASP-attb

Required Primers

---	CAGATCCACTAGTGGCCTATG	pUASP-attb	Rev	62.3°C	64.6°C	view

pUASP-attb	ccgcggccgcataggccactagtggatctgGTTTCATTCGGTTTCGTG	5'_fragment	Fwd	56.4°C	58.6°C	view
3'_fragment_with_3x_flag	gccgccgatgcattggGATTGTTCAGATCAATCTCG	5'_fragment	Rev	55.6°C	58.6°C	view
5'_fragment	ttgatctgaacaatcCCAATGCATCGGCGGCAA	3'_fragment_with_3x_flag	Fwd	67.6°C	66.0°C	view
pUASP-attb	taacgttaacgttcgaggtcgactctagagCTATCACTTGTCGTCGTCGTC	3'_fragment_with_3x_flag	Rev	63.0°C	66.0°C	view
---	CTCTAGAGTCGACCTCGAAC	pUASP-attb	Fwd	61.6°C	64.6°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- A minimum overlap of 15 nt should be sufficient for the assembly of less than 4 fragments in 1 reaction. The current setting is 30. This setting can be adjusted in the Set Preferences tab.

Assembled Sequence

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HiFi DNA Assembly® Protocol

Optimal Quantities
NEB recommends a total of 0.03–0.2 pmols of DNA fragments when 1 or 2 fragments are being assembled into a vector and 0.2–0.5 pmoles of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

50 ng of 5000 bp dsDNA is about 0.015 pmols.

50 ng of 500 bp dsDNA is about 0.15 pmols.

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Assembly Protocol

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly*	4-6 Fragment Assembly**	Positive Control†
Recommended DNA Ratio	vector:insert = 1:2	vector:insert = 1:1	
Total Amount of Fragments	0.03–0.2 pmols* X µl	0.2–0.5 pmols* X µl	10 µl

Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

2. * Optimized cloning efficiency is 50–100 ng of vectors with 2 fold excess of inserts. Use 5 times more inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.
   ** To achieve optimal assembly efficiency, it is recommended to design ≥ 20 bp overlap regions between each fragment with equimolarity (suggested: 0.05 pmol each).
   † Control reagents are provided for 5 experiments.
   ‡ If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional Assembly Master Mix.
3. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

   Note: Reaction times less than 15 minutes are generally not recommended. Extended incubation times (up to 4 hours) have been shown to improve assembly efficiencies in some cases. Do not incubate the assembly reaction overnight.

4. Transform NEB 5-alpha Competent E. *coli* cells (provided in the cloning kit or purchased separately from NEB) with 2 µl of the assembled product, following the appropriate transformation protocol.

Transformation Protocols

Transformation with chemically competent cells.

1. Thaw chemically competent cells on ice.

2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube (if necessary).

3. If the chemically competent cells are from New England Biolabs, add 2 µl of assembled product to NEB competent cells and go to step 4 directly. If competent cells are purchased from other manufacture, dilute assembled products 4-fold with H_2O prior transformation. This can be achieved by mixing 5 µl of assembled products with 15 µl of H_2O. Add 2 µl of the diluted assembled product to competent cells.

4. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix.

5. Heat shock at 42°C for 30 seconds.* Do not mix.

6. Transfer tubes on ice for 2 minutes.

7. Add 950 µl of room temperature SOC media* to tubes.

8. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.

9. Warm selection plates to 37°C.

10. Spread 100 µl of the cells onto the plates with appropriate antibiotics. Use Amp plates for positive control sample.

11. Incubate plates overnight at 37°C.

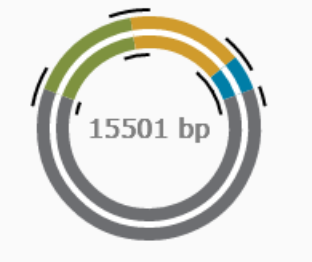
    * Please note: Follow the manufacturer's protocols for the duration and temperature of the heat shock step, as well as the optimal medium for recovery. Typically, transformation of our positive control assembly product will yield more than 100 colonies on an Amp plate with greater than 80% colonies containing inserts.

    NEB recommends NEB 5-alpha Competent *E. coli* (NEB #C2987) for transformation of Gibson Assembly products. It is also possible to use other NEB competent *E. coli* strains, with the exception of BL21, BL21(DE3), Lemo21(DE3) and Nico21(DE3). For example, Shuffle T7 Express Competent *E. coli* can be used for the expression of a difficult to express protein. When using competent *E. coli* from a vendor other than NEB, we have seen decreased robustness of transformation with the Gibson Assembly reaction.

Transformation with electrocompetent cells.

1. Thaw electrocompetent cells on ice.
2. Transfer 50 µl of electrocompetent cells to a pre-chilled electroporation cuvette with 1 mM gap.
3. Dilute assembled products 3-fold with H_2O prior electroporation. This can be achieved by mixing 5 µl of assembled products with 10 µl of H_2O. Add 1 µl of the diluted assembly product to electrocompetent cells.
4. Mix gently by pipetting up.
5. Once DNA is added to the cells, electroporation can be carried out immediately. It is not necessary to incubate DNA with cells.
6. Add 950 µl of room temperature SOC media to the cuvette immediately after electroporation.
7. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the plates.
10. Incubate overnight at 37°C.

Act5c promoter:

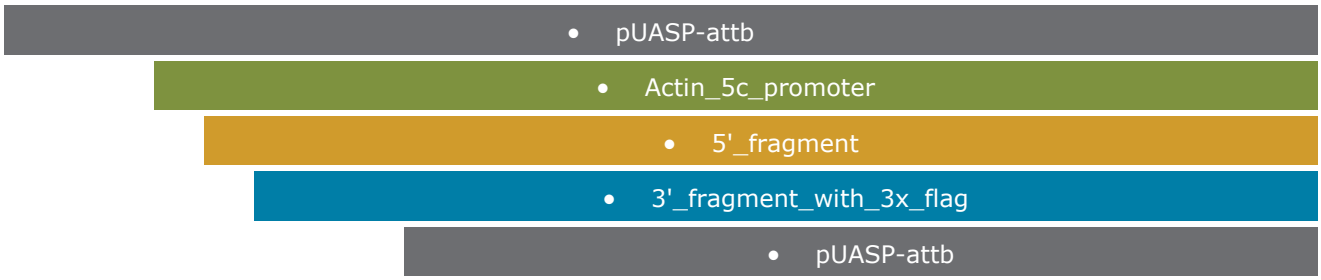


User Selected Settings

Product Version	E2611 - Gibson Assembly Master Mix

No. of Fragments	4-6 fragments (including vector)
Min. Overlap	30 bp
Min. Fragment Length	150 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement



Required Primers

---	CTAGTATGTATGTAAGTTAATAAAACCC	pUASP-attb	Rev	56.9°C	59.9°C	view
pUASP-attb	gggttttattaacttacatacatactagGCATGCAATTCTATATTCTAAAAACACAAATG	Actin_5c_promoter	Fwd	modified	modified	view
5'_fragment	gaaaccgaatgaaacATCTGGATCCGGGGTCTC	Actin_5c_promoter	Rev	63.8°C	64.7°C	view
Actin_5c_promoter	accccggatccagatGTTTCATTCGGTTTCGTG	5'_fragment	Fwd	56.4°C	58.6°C	view
3'_fragment_with_3x_flag	gccgccgatgcattggGATTGTTCAGATCAATCTCG	5'_fragment	Rev	55.6°C	58.6°C	view
5'_fragment	ttgatctgaacaatcCCAATGCATCGGCGGCAA	3'_fragment_w	Fw	67.6°	66.0°	vie

		ith_3x_flag	d	C	C	w
pUASP-attb	taacgttaacgttcgaggtcgactctagagCTATCAC TTGTCGTCGTCGTC	3'_fragment_with_3x_flag	Rev	63.0° C	66.0° C	view
---	CTCTAGAGTCGACCTCGAACG	pUASP-attb	Fwd	64.4° C	59.9° C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.

Assembled Sequence

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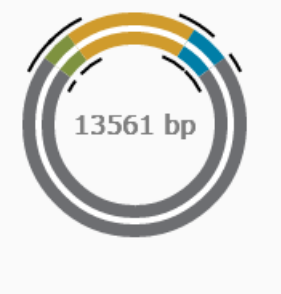
Hsp70 promoter:

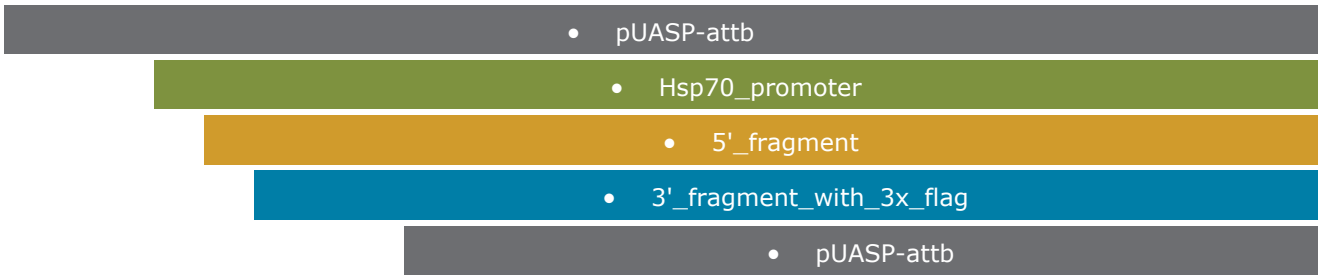


User Selected Settings

Product Version	E2611 - Gibson Assembly Master Mix
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	30 bp

Min. Fragment Length	150 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement



Required Primers

---	CTAGTATGTATGTAAGTTAATAAAACCC	pUASP-attb	Rev	56.9°C	59.9°C	view
pUASP-attb	atgggttttattaacttacatacatactagCATGCTGGAGCTATCCGC	Hsp70_promoter	Fwd	63.9°C	64.1°C	view
5'_fragment	gaaaccgaatgaaacTTTGCTCAGCTTGCTTCG	Hsp70_promoter	Rev	61.1°C	64.1°C	view
Hsp70_promoter	agcaagctgagcaaaGTTTCATTCGGTTTCGTG	5'_fragment	Fwd	56.4°C	58.6°C	view
3'_fragment_with_3x_flag	gccgccgatgcattggGATTGTTCAGATCAATCTCG	5'_fragment	Rev	55.6°C	58.6°C	view
5'_fragment	ttgatctgaacaatcCCAATGCATCGGCGGCAA	3'_fragment_with_3x_flag	Fwd	67.6°C	66.0°C	view

pUASP-attb	taacgttaacgttcgaggtcgactctagagCTATC ACTTGTCGTCGTCGTC	3'_fragment_with_3x_flag	Rev	63.0°C	66.0°C	view
---	CTCTAGAGTCGACCTCGAACG	pUASP-attb	Fwd	64.4°C	59.9°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.

Assembled Sequence

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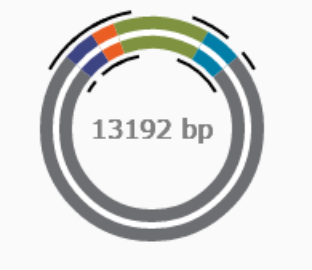

User Selected Settings

Product Version	E2611 - Gibson Assembly Master Mix
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	30 bp
Min. Fragment Length	150 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Fragment Arrangement

- PUASP-attb
- Hsp70_promoter
- 5'_and_first_21aminoacids
- Fragment_from_synthesized_5'end
- 3’end_fragment_without_flag
- PUASP-attb

Required Primers

---	CTAGTATGTATGTAAGTTAATAAAACCC	PUASP-attb	Rev	56.9°C	59.9°C	view
PUASP-attb	atgggttttattaacttacatacatactagCATGCTGGAGCTATCCGC	Hsp70_promoter	Fwd	63.9°C	64.1°C	view
5'_and_first_21aminoacids	gaaaccgaatgaaacTTTGCTCAGCTTGCTTCG	Hsp70_promoter	Rev	61.1°C	64.1°C	view
Hsp70_promoter	agcaagctgagcaaaGTTTCATTCGGTTTCGTG	5'_and_first_21aminoacids	Fwd	56.4°C	59.4°C	view
Fragment_from_synthesized_5'end	cgccgccagtgcgattGATCAGTGTGCTCTGGTG	5'_and_first_21aminoacids	Rev	60.5°C	59.4°C	view
5'_and_first_21aminoacids	cagagcacactgatcAATCGCACTGGCGGCGGA	Fragment_from_synthesized_5'end	Fwd	71.5°C	70.9°C	view
3’end_fragment_without_flag	gccgccgatgcattggGATTGTTCAGATCAATCTCGCTAATCTCATACTTGG	Fragment_from_synthesized_5'end	Rev	67.9°C	70.9°C	view
Fragment_from_synthesized_5'end	ttgatctgaacaatcCCAATGCATCGGCGGCAA	3’end_fragment_without_flag	Fwd	67.6°C	69.2°C	view
PUASP-attb	taacgttaacgttcgaggtcgactctagagCTAGACCTTGCGGCCTCG	3’end_fragment_without_flag	Rev	66.2°C	69.2°C	view
---	CTCTAGAGTCGACCTCGAAC	PUASP-attb	Fwd	61.6°C	59.9°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are

assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.

Assembled Sequence

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Protocol for Q5® High-Fidelity 2X Master Mix

Please note that protocols with Q5 High-Fidelity DNA Polymerase may differ from protocols with other polymerases. Conditions recommended below should be used for optimal performance.

Reaction Setup:

We recommend assembling all reaction components on ice and quickly transferring the reactions to a

thermocycler preheated to the denaturation temperature (98°C). All components should be mixed prior to use.

Component	25 µl Reaction	50 µl Reaction	Final Concentration
Q5 High-Fidelity 2X Master Mix	12.5 µl	25 µl	1X
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 1,000 ng
Nuclease-Free Water	to 25 µl	to 50 µl	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes to a PCR machine and begin thermocycling.

Thermocycling Conditions for a Routine PCR:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	*50–72°C	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

*Use of the NEB T_m Calculator is highly recommended.

General Guidelines:

1. Template:
   Use of high quality, purified DNA templates greatly enhances the success of PCR. Recommended amounts of DNA template for a 50 µl reaction are as follows:

DNA	AMOUNT
DNA Genomic	1 ng–1 µg
Plasmid or Viral	1 pg–1 ng

2. Primers:
   Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Computer programs such as Primer3 can be used to design or analyze primers. The best results are typically seen when using each primer at a final concentration of 0.5 µM in the reaction.

3. Mg^{++} and additives:
   The Q5 High-Fidelity Master Mix contains 2.0 mM Mg^{++} when used at a 1X concentration. This is optimal for most PCR products generated with this master mix.

4. Deoxynucleotides:
   The final concentration of dNTPs is 200 µM of each deoxynucleotide in the 1X Q5 High-Fidelity Master Mix. Q5 High-Fidelity DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates.

5. Q5 High-Fidelity DNA Polymerase concentration:
   The concentration of Q5 High-Fidelity DNA Polymerase in the Q5 High-Fidelity 2X Master Mix has been optimized for best results under a wide range of conditions.

6. Denaturation:
   An initial denaturation of 30 seconds at 98°C is sufficient for most amplicons from pure DNA templates. Longer denaturation times can be used (up to 3 minutes) for templates that require it.

   During thermocycling, the denaturation step should be kept to a minimum. Typically, a 5–10 second denaturation at 98°C is recommended for most templates.

7. Annealing:
   Optimal annealing temperatures for Q5 High-Fidelity DNA Polymerase tend to be higher than for other PCR polymerases. The **NEB T_m Calculator** should be used to determine the annealing temperature when using this enzyme. Typically use a 10–30 second annealing step at 3°C above the T_m of the lower T_m primer. A temperature gradient can also be used to optimize the annealing temperature for each primer pair.

For high T_m primer pairs, two-step cycling without a separate annealing step can be used (see note 10).

8. Extension:
   The recommended extension temperature is 72°C. Extension times are generally 20–30 seconds per kb for complex, genomic samples, but can be reduced to 10 seconds per kb for simple templates (plasmid, *E. coli*, etc.) or complex templates < 1 kb. Extension time can be increased to 40 seconds per kb for cDNA or long, complex templates, if necessary.

   A final extension of 2 minutes at 72°C is recommended.

9. Cycle number:
   Generally, 25–35 cycles yield sufficient product. For genomic amplicons, 30-35 cycles are recommended.

10. 2-step PCR:
    When primers with annealing temperatures ≥ 72°C are used, a 2-step thermocycling protocol (combining annealing and extension into one step) is possible.

11. Amplification of long products:
    When amplifying products > 6 kb, it is often helpful to increase the extension time to 40–50 seconds/kb.

12. PCR product:
    The PCR products generated using Q5 High-Fidelity 2X Master Mix have blunt ends. If cloning is the next step, then blunt-end cloning is recommended. If T/A-cloning is preferred, the DNA should be purified prior to A-addition, as Q5 High-Fidelity DNA Polymerase will degrade any overhangs generated.

    Addition of an untemplated -dA can be done with *Taq* DNA Polymerase (NEB #M0267) or Klenow exo$^-$ (NEB #M0212).