**BROCCOLI AND dBROCCOLI PLASMIDS**

Broccoli is noticeably better than Spinach2 for imaging RNA in *E. coli* and mammalian cells. One of the reasons of superior behavior of Broccoli in cells is low dependence of its fluorescence on magnesium concentration.

An additional tag is dimeric Broccoli (dBroccoli) which is two Broccoli aptamers within one long stem-loop. dBroccoli is almost two times brighter than Broccoli *in vitro* and *in vivo* while still having relatively small size (90 nt).

We have developed a new scaffold, F30, which enhances Broccoli fluorescence *in vitro* and in cells. F30 was engineered based on the naturally occurring Phi29 viral RNA three-way junction motif. Unlike the previously used tRNA scaffold, F30 is not recognized by cellular nucleases and thus is not cleaved. This indicates that F30 is a bioorthogonal scaffold in mammalian cells and thus it can be used as a tag when presenting RNA aptamers. F30-Broccoli was shown to be substantially brighter than unscaffolded Broccoli both in bacterial and mammalian cells.

F30 has two entry points for inserting aptamers. We inserted dBroccoli into each entry point to create F30-2xdBroccoli. Thus, this F30 scaffold presents the equivalent of four Broccoli units and indeed is roughly four times brighter than F30-Broccoli in cells.

Broccoli and dBroccoli were described in 2014: Filonov, G.S., Moon, J.D., Svensen, N., Jaffrey, S.R.Broccoli: rapid selection of an RNA mimic of green fluorescence protein by fluorescence-based selection and directed evolution, *JACS*, 2014, 136 (46), pp 16299–16308.

F30-Broccoli and F30-2xdBroccoli tags were described in 2015: Filonov, G.S., Kam, K.W., Song, W., Jaffrey, S.R. In-gel imaging of RNA processing using Broccoli reveals optimal aptamer expression strategies, Chemistry & Biology, 22, pp 649–660.

The sequence of the F30-Broccoli gene is as follows:

UUGCCAUGUGUAUGUGGGAGACGGUCGGGUCCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUCCCACAUACUCUGAUGAUCCUUCGGGAUCAUUCAUGGCAA

The Broccoli sequence is indicated in green, while the F30 scaffold sequence is in orange.

The sequence of the F30-2xdBroccoli gene is as follows:

UUGCCAUGUGUAUGUGGGAGACGGUCGGGUCCAUCUGAGACGGUCGGGUCCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUCAGAUGUCGAGUAGAGUGUGGGCUCCCACAUACUCUGAUGAUCCAGACGGUCGGGUCCAUCUGAGACGGUCGGGUCCAGAUAUUCGUAUCUGUCGAGUAGAGUGUGGGCUCAGAUGUCGAGUAGAGUGUGGGCUGGAUCAUUCAUGGCAA

The dBroccoli sequences are in green, while the F30 scaffold sequence is in orange.

You can PCR this out and put it in whatever plasmid you want. You can also PCR this construct and include a T7 promoter for *in vitro* transcription off of the PCR product.

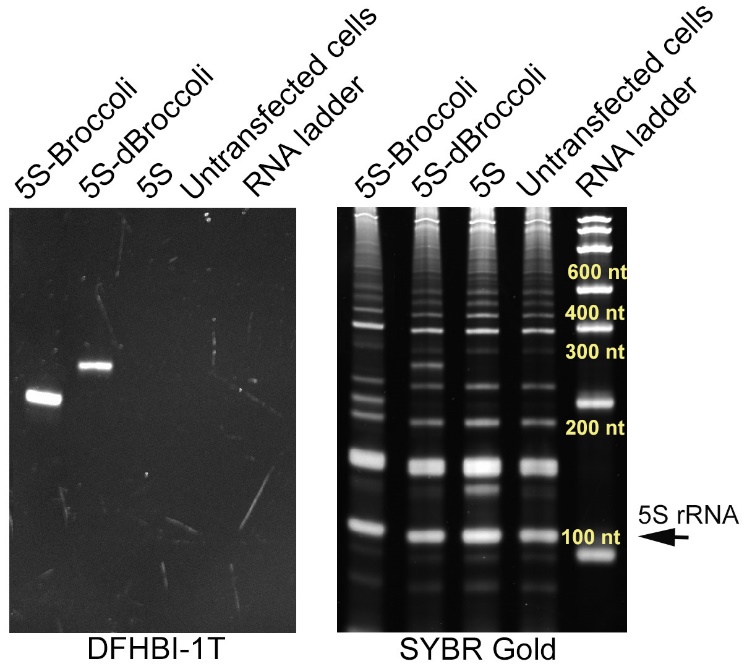
Before you tag an RNA with Broccoli or dBroccoli for mammalian or *E. coli* expression, we recommend that you insert Broccoli or dBroccoli into the desired sequence and confirm that the aptamer remains properly folded and is fluorescent. Aptamers can fold improperly due to hybridization with adjacent sequences. The RNA can be cloned into a construct that contains a T7 promoter and used for *in vitro* transcription. Use F30-Broccoli itself as a positive control.

You should run a gel to ensure that the *in vitro* transcribed RNA is of proper length (sometimes *in vitro* transcription gives degraded products or incorrect RNA). The gel can be stained with any conventional dye (SYBR Green, Gold etc.) or with DFHBI to specifically visualize Broccoli fluorescence (we typically stain gels with DFHBI first, then SYBR gold afterwards). Either native or denaturing PAGE can be used. The staining protocol is as follows: After the gel is run to completion, wash the gel 3x5 min with water and then stain for 15-30 min with 10 µM DFHBI in 40 mM HEPES pH 7.4, 100 mM KCl, and 1 mM MgCl2. Image the gel using blue light excitation and cyan-blue light emission. We use a ChemiDoc MP instrument with 470±15 nm excitation and 532±14 nm emission. If your Broccoli signal is strong, you should also be able to detect it with your naked eye under the UV excitation. Then this gel can be washed to remove DFHBI (3x5 min with water) and stained with RNA-specific dye of your choice according to the manufacturer’s protocol (we use SYBR Gold). The detailed protocol can be found in Filonov et al., Chemistry and Biology, 2015.

To measure the fluorescence of your *in vitro* synthesized Broccoli-tagged RNA, the final buffer should contain 40 mM HEPES pH 7.4, 100 mM KCl, and 1 mM MgCl2, 1 µM Broccoli (or test construct, or water only control), and 10 µM DFHBI (this can be obtained from Lucerna Technologies: <http://www.lucernatechnologies.com/>). (Other fluorophores are available—see Notes at the end of this document.) Subtract fluorescence signal from DFHBI sample with no RNA from all measurements. In general the Broccoli-tagged RNA should have between 50%-100% of the Broccoli fluorescence signal before you proceed and clone the construct into an expression vector for mammalian expression.

After you confirm that Broccoli fused to your RNA remains fluorescent you can proceed to *in vivo* experiments. Our routine procedure to study Broccoli-tagged RNAs *in vivo* is as follows:

First we make sure that our Broccoli-tagged RNA is properly expressed in cells. To do this, we use total cellular RNA separation on urea-PAGE followed by the above-mentioned gel staining protocol with DFHBI, which detects Broccoli very specifically. In the example below, total RNA was isolated using Trizol LS reagent from the HEK293T cells transfected with the plasmids encoding either 5S-Broccoli or 5S-dBroccoli. Then 5 µg of total RNA was loaded into each well of precast 6% TBE-urea PAGE gel (Life Technologies) and ran at 270-300 V in 1x TBE buffer. RiboRuler Low Range RNA Ladder (Thermo Scientific) was used as molecular weight standard. Again, after the gel is run to completion, wash the gel 3x5 min with water and then stain for 15-30 min with 10 µM DFHBI in 40 mM HEPES pH 7.4, 100 mM KCl, and 1 mM MgCl2. Image the gel with the blue light excitation and cyan-green light emission. Then this gel can be washed to remove DFHBI (3x5 min with water), and then stained with RNA-specific dye of your choice according to the manufacturer’s protocol. We use SYBR Gold.



After you confirm proper expression of your Broccoli-tagged RNA with the gel staining you can proceed to imaging experiments. Here is an example of 5S labeled with a Broccoli and dBroccoli at the 3’ end. No tRNA scaffold was used. This was imaged at 500 ms using a GFP filter cube on an epifluorescence microscope (Filonov *et al.*, JACS, 2014). The cells were pretreated with 0.3 M sucrose to induce stress granule formation.

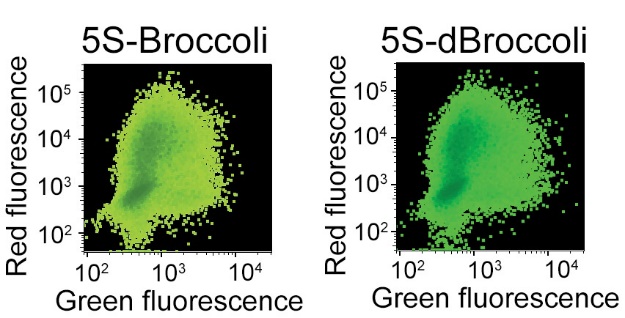




Typically to detect 5S-Broccoli labeling in sucrose-untreated cells, we recommend that you subtract background levels of fluorescence. This allows you to tell which cells are expressing 5S-Broccoli. Keep in mind that the signal will bleach rapidly, so continuous light exposure will harm your imaging.

This Addgene submission has 5S-F30-2xdBroccoli-expressing plasmid, which shows even brighter signal when expressed in mammalian cells.

Another way to detect Broccoli or Broccoli-labeled RNA in cells is to use flow cytometry. Below are FACS analysis dot plots of HEK293T cells transfected with 5S-Broccoli or 5S-dBroccoli plasmids. mCherry was expressed from a cotransfected plasmid to serve as a transfection efficiency control. Broccoli signal was detected in the green channel (ex=488 nm, em= 525±25 nm), mCherry signal was detected in the red channel (ex=561 nm, em=610±10). Flow cytometry can be a good way to assess your RNA-Broccoli brightness on a whole population level. 5S-Broccoli signal can be easily detected even without treatment with sucrose. Flow cytometry analysis of Broccoli or dBroccoli expression in cells is also advantageous because flow cytometry detection is very fast and thus is not impaired by the rapid photobleaching of Broccoli or dBroccoli.



**HEK293T cell imaging protocol (see Paige et al., 2011 for more details)**

**A. Preparing HEK293T cells for imaging**

1. 24-well glass-bottom dishes (Mat Tek Corp – Cat # P24G-1.5-13F) are briefly zapped with a hand-held corona discharger and coated with 300 μL of poly-L-lysine (PLL) overnight at 4°C.

2. Next morning, the dishes are washed once with ddH2O and UV sterilized for 5 min. The dishes are further coated with 50 μL of rat collagen-I and laminin (1:100) for 2 hours at 37°C.

3. After removing the coating solution by washing 2X with 1 ml dH2O, HEK293T cells are plated at 80,000 cells/ml. Grow cells in DMEM + 10% FBS without dye (Life Technologies – Cat # 31053036).

4. HEK293T cells are transfected with 0.4 μg of mammalian expression plasmid with Broccoli (dBroccoli) the next day (25-40% confluent) with FuGeneHD (Promega – Cat # E2311). [0.6 μg DNA, 1.65 μL FuGeneHD, in dH2O => 25 μL/well] Imaging experiments are performed 48 hours after transfection. High level transfection is important for robust signals.

**B. Imaging Broccoli**

5. Thirty minutes prior to experiment, replace HEK293 cell media with prewarmed imaging media (DMEM with no phenol red or vitamins and supplemented with 25 mM HEPES pH 7.4 and 5 μg/ml Hoechst 33342). Also add 20 μM DFHBI/DFHBI-1T (dilute from 40 mM stock in DMSO) to the cells to promote RNA-fluorophore complex formation. In control wells, add only DMSO.

6. To induce stress granule formation in cells transfected with 5S-F30-2xdBroccoli, treat cells with 300 mM sucrose immediately prior to imaging. Look for cells that are not too bunched together and relatively flat. You can also use up to 600 mM sucrose, although there may be some cytotoxicity. Since it is difficult to make a sucrose stock solution, we simply make DMEM with 300 mM sucrose and switch from the normal sucrose-free media to the sucrose-containing media. Observe stress granules formation after 5-20 minutes of treatment and up to 40 minutes after that.

7. Live fluorescence images of HEK293T cells are taken with a CoolSnap HQ2 CCD camera through a 60X oil objective (Plan Apo 1.4 NA) mounted on a Nikon TE2000 epifluorescence microscope and analyzed with the NIS-Elements software.

8. DFHBI fluorescence is detected using the FITC filter (0.5-1 s exposure), Hoescht 33342 fluorescence with the DAPI filter (~100 ms exposure) and phase-contract images are taken at ~50 ms.

9. Using your imaging software, take images of untransfected control cells with DFHBI and subtract the background so that any green autofluorescence or residual fluorescence from DFHBI is eliminated. Use these settings to subtract background from Broccoli-containing cells. This is your Broccoli signal.



**pET28c-F30-Broccoli and pET28c-F30-2xdBroccoli plasmids for imaging Broccoli in *E. coli*:**

Broccoli (or 2xdBroccoli) within the F30 scaffold was cloned between T7 promoter and T7 terminator sequences of pET28c.

**To express and visualize Broccoli in *E. coli*:**

1. We have found the most robust expression in BL21 Star (DE3) *E. coli* cells (Invitrogen). To express in BL21s, we transform cells with 40 ng of pET28c-Broccoli plasmid.
2. Cells are plated, incubated overnight in a 37oC incubator, and single colonies are picked for inoculation in Luria Broth containing kanamycin (LB-Kan).
3. At OD600 = 0.4, 1 mM of IPTG was added to the culture and shaking was continued at 37oC for 2 more hours. We found 2 hours to be the optimal amount of time to maximize Broccoli signal. Longer IPTG incubations did not increase Broccoli fluorescence.
4. 100 µL of culture was then removed, spun down to pellet the culture, and resuspended in 2 mL of pH 7.0 M9 minimal media or PBS media.
5. A 200 µl aliquot of resuspended culture was then plated on poly-L-lysine (PLL)-coated 24-well glass-bottom dishes (MatTek) and incubated for 45 minutes at 37oC. This provides time to allow *E. coli* to stick to the glass.
6. Adherent cells were washed twice and then incubated with 200 µM DFHBI in PBS or pH 7.0 M9 minimal media for 45 min at 37oC. We noticed that for Spinach2 lowering the pH to 6.5 or 6.0 increases the fluorescence signal in cells. This may be true for Broccoli as well though we have not tested that. DFHBI can be purchased from commercial suppliers. See [www.jaffreylab.com](http://www.jaffreylab.com), and click the reagents link, to find the latest information and vendors.
7. Live fluorescence images were taken with a CoolSnap HQ2 CCD camera through a 60X oil objective mounted on a Nikon TE2000 microscope and analyzed with the NIS-Elements software. We use a GFP filter cube: excitation filter 470/40, dichroic mirror 495 (long pass) and emission filter 525/50 (Chroma Technology).
8. We confirmed that fluorescence can be readily detected within a temperature range of 28-40oC. Higher or lower temperatures may work as well but we have not confirmed this yet. Fluorescence may be slightly brighter at lower temperatures due to better Broccoli folding.

**Notes**:

1. Initially Broccoli plasmids for bacterial and mammalian expression were made so that Broccoli also contained the tRNA scaffold. However we have found that tRNA-scaffolded Broccoli is recognized as tRNA in cells and subjected to ribonucleolytic cleavage. Thus, tagging your RNA with aptamers within the tRNA scaffold may not be optimal. Because of this, we engineered the F30 scaffold. This scaffold provides brighter Broccoli signal and is superior for bacterial and mammalian expression since it has no or minimal amount of unwanted shorter transcripts resulted from the full-length transcript cleavage or premature transcription termination. We advise you first to try fusing your RNA to Broccoli (2xdBroccoli) within F30 and testing it in cells using both microscopy and the gel staining approach.

2. If you use the 5S promoter, please cite Paul et al., Localized Expression of Small RNA Inhibitors in Human Cells, Molecular Therapy, 7:237-247, 2003, which describes this expression system.

3. We tagged 5S RNA for imaging Broccoli-tagged RNA in mammalian cells. 5S is very abundant. To image lower abundance RNAs, you may need to put tandem copies of Broccoli or dBroccoli. This is something we have not thoroughly tested, although we have already shown that two F30 scaffolds with Broccoli aptamers can be fused to from a F30-F30 cassette with enhanced brightness. Conceivably F30-2xdBroccoli, which is so far the brightest tag we have, can be used to engineer a cassette with two or more F30-dxBroccoli in a row. However, you should ensure that insertion of Broccoli does not induce degradation of your transcript, which is sometimes a consequence of inserting aptamers in RNAs. We find that aptamers inserted after the stop codon have the least destabilizing effects.

4. Fluorophores are available from commercial sources. We are actively trying to find distributors for fluorophores, including distributors in Asia. Currently, Lucerna Technologies in the USA (<http://www.lucernatechnologies.com/>) distributes the following fluorophores:

DFHBI

DFHBI-1T

Which fluorophore should you use?

DFHBI is probably sufficient for most work in *E. coli*, and in mammalian cells for RNAs that are highly expressed or in large aggregates (like CGG-repeat RNA and 7SK, etc). DFHBI-1T has certain important advantages (see Song, W., Strack, R.L., Svensen, N., Jaffrey, S.R. Plug-and-play fluorophores extend the spectral properties of Spinach. Journal of the American Chemical Society, advanced online, 2014, <http://pubs.acs.org/doi/abs/10.1021/ja410819x>). These advantages are lower background fluorescence from nonspecific binding of the fluorophore to cellular components, and excitation and emission spectra that more closely match that of GFP. Broccoli-DFHBI complex has an Exmax = 447 nm and Emmax = 501 nm, while Broccoli-DFHBI-1T is Exmax = 472 nm and Emmax = 507 nm. You can see that the excitation of Broccoli-DFHBI-1T is optimal for most fluorescence microscopes when you image using the FITC/GFP channel.

As a result of better excitation of the fluorophore, the images show brighter Broccoli-specific signals (You can see this shown for Spinach2 in our JACS paper: <http://pubs.acs.org/articlesonrequest/AOR-TugsAwf95q3PncFPvWcd> ). Typically the images are ~40% brighter. Additionally, because of lower background signals, you can use longer exposure times. Both these factors result in enhanced sensitivity. The drawback is that DFHBI-1T is likely to be more expensive due to its synthetic complexity.

**Appendix 1.**

Part of the pAV5S-F30-2xdBroccoli plasmid map with the 5S and F30-2xdBroccoli sequences.

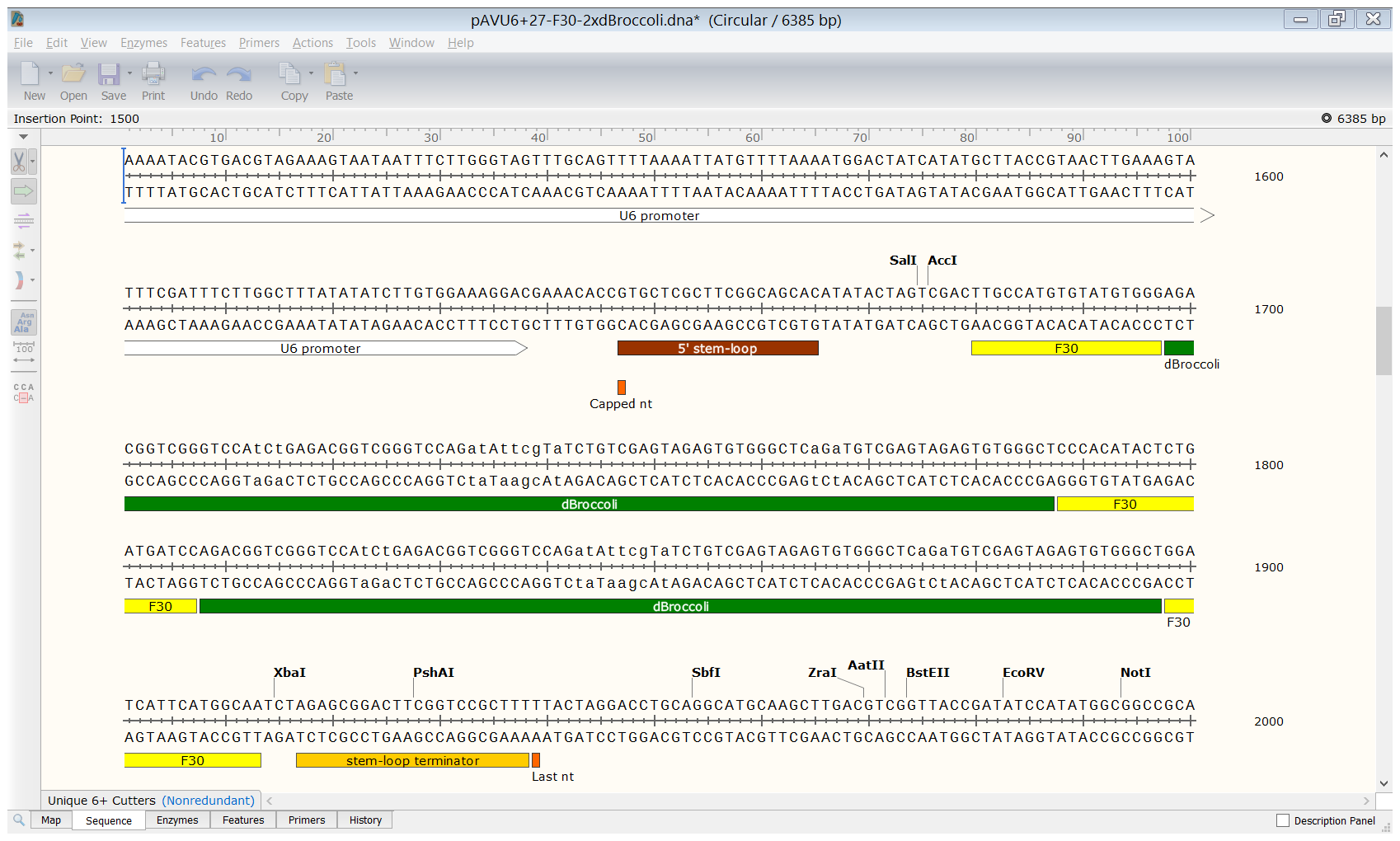
*Sal*I and *Xba*I were used for cloning. Restriction enzymes in bold are unique in the plasmid.



**Appendix 2.**

Part of the pAVU6+27-F30-2xdBroccoli plasmid map with the U6 and F30-2xdBroccoli sequences.

*Sal*I and *Xba*I were used for cloning. Restriction enzymes in bold are unique in the plasmid.



**Appendix 3.**

Part of the pET28c-F30-Broccoli plasmid map with F30-Broccoli and T7 promoter and terminator.

*Bgl*II and *Xho*I were used for cloning. Restriction enzymes in bold are unique in the plasmid.



**Appendix 4.**

Part of the pET28c-F30-2xdBroccoli plasmid map with F30-2xdBroccoli and T7 promoter and terminator. *Bgl*II and *Xho*I were used for cloning. Restriction enzymes in bold are unique in the plasmid.

