

In vitro transcription of spike controls from T7-dT PCR Products

Outline

To make RNA from each of the spike control clones, specific primer pairs were designed for each spike to PCR amplify between 0.3 to 1.5 kb of each. The primers were 5' end modified to contain the T7 promoter sequence and 3' end modified to contain a 15mer poly T tail. The T7-dT amplified DNA for each spike was then transcribed into RNA using the Ambion MEGAscript T7 kit ([Ambion](#)). These spikes were then mixed and added to each labelling reaction.

PCR Amplification with 5'-T7 and dT₁₅-3' primers

PCR primers:

Please note that '[T7]' refers to the nucleotide sequence 'TAATACGACTCACTATAGGGAGA'.

Clone	CloneID	5'-T7 primer	3'-dT ₁₅ primer	RNA length (bp)
Arizona 4	M90509	[T7]-gaaccagtgataggttcttg	(T) ₁₅ atagcatgctcgatgtgcaa	510
Arizona 6	U74610	[T7]-tcctctcttctcaacctcg	(T) ₁₅ acaacggaagcaaatcttattg	942
Incyte 4	ATU18126	[T7]-tcaaaagcttcgaatctggc	(T) ₁₅ aaggttgcaggttattcttc	517
Incyte 5	L22585	[T7]-agctcaatggtcactatgatg	(T) ₁₅ cgctaggcatgcttaaataacc	489
AIMS 1	AB007987	[T7]-agatgcttctctctctc	(T) ₁₅ tggttgtaatggcttaccg	1151
AIMS 4	AF117335	[T7]-agtggatgaggaataggagc	(T) ₁₅ taccatactggatccttccc	1540
AIMS 5	AF168390	[T7]-gatattcccgtgtctctc	(T) ₁₅ tgaccataagccaactgcatc	1157
AIMS 9	AF372915	[T7]-agatcatcctcataggcgatg	(T) ₁₅ aagcgaagaagctctgggc	1102
AIMS 10	Y18469	[T7]-agtgctgctactcactggg	(T) ₁₅ tgagataactagagaaggtcc	1405
AIMS 11	Z49777	[T7]-actaaacatggcgacggag	(T) ₁₅ aaactagcgcgtcatggtgg	987
AIMS 19	X644464	[T7]-tgggtaaagctggctgcaagg	(T) ₁₅ accgaaaatagcaatccgacc	775
Weed 1	O82258	[T7]-taaagtggaacctccgatgc	(T) ₁₅ gaagagctcatcgccgatac	514
Weed 3	Q9LJQ4	[T7]-ttctcacaactcgtaattcaa	(T) ₁₅ gcaactgatgaccaggaaga	402
Weed 4	Q9XIB8	[T7]-aagacgagcgagatctca	(T) ₁₅ tggtccttcagagtgcaaatg	396
Weed 6	O04600	[T7]-ttgagtaccaacggttcagc	(T) ₁₅ tatcatcggttgcctttgc	370
Weed 7	Q9LZJ2	[T7]-tcatgtgaacatacaacgcaat	(T) ₁₅ ggtctattgggggtggaatc	404
Weed 8	Q9LVF8	[T7]-tcaacctatcattcctccatt	(T) ₁₅ gcctattgaggatttgttctt	394
Weed 9	O49366	[T7]-agcttgagaacataggccaca	(T) ₁₅ tggtcatcggttctctctgta	343
Weed 10	O81842	[T7]-agcatcaaaatccaacaa	(T) ₁₅ ttcgattccgcagattatcc	361

Weed 13	Q9LU32	[T7]-tccaatatgattggttgga	(T) ₁₅ tgtatgcttcactcgatga	330
Weed 14	O04513	[T7]-agggcatttggttcaggt	(T) ₁₅ atagcatgctcgatgtgcaa	306

PCR reaction mix:

- 10 µl 10 x Stratagene Yield Ace reaction buffer
- 2 µl 10 mM dNTP
- 84 µl MilliQ water
- 1 µl Stratagene Yield Ace DNA polymerase
- 1 µl plasmid DNA
- 2 µl 25 pmol / µl of 5'-T7 and dT₁₅-3' primer pairs

PCR cycle:

All PCR reactions were performed in 0.2 ml microfuge tubes with a Dyad thermal cycler with the following PCR cycle.

1. 94 °C for 3 minutes
2. 94 °C for 30 seconds
3. 60 °C for 30 seconds
4. 72 °C for 4 minutes
5. Repeat steps 2 to 4 34 times
6. 72 °C for 10 minutes
7. 4 °C cold storage before unloading

The PCR products were purified by QIAquick spin columns and checked by agarose gel electrophoresis.

***In vitro* transcription reaction from 5'-T7 and dT₁₅-3' PCR templates**

Protocol for the Ambion MEGAscript T7 kit:

1. Prepare the following *in vitro* transcription reaction mix:
 - ◆ 7 µl Ambion nuclease-free water
 - ◆ 2 µl dATP
 - ◆ 2 µl dUTP
 - ◆ 2 µl dGTP
 - ◆ 2 µl dCTP
 - ◆ 2 µl 10 x Reaction Mix
 - ◆ 1 µl DNA Template (5'-T7 and dT₁₅-3' PCR product)
 - ◆ 2 µl T7 polymerase
2. Incubate at 37 °C for 2 to 4 hours
3. Perform a DNase treatment:
 - ◆ Add 1ul DNase
 - ◆ Mix with a pipette
 - ◆ Pulse spin to collect contents to bottom of tube
 - ◆ Incubate 37 °C for 15 minutes
4. Stop Reaction and precipitate RNA:
 - ◆ 30ul Nuclease-free water (from kit)
 - ◆ 25ul Lithium Chloride solution (from kit)
 - ◆ Mix and freeze at -20 °C for at least 30 minutes
 - ◆ Spin at 13,000 rpm (RT or 4 °C) for 15 minutes to pellet RNA
 - ◆ Wash pellet in 1 ml 70% ethanol (made with DEPC water)
 - ◆ Spin for 5 minutes at 13000 rpm
 - ◆ Resuspend RNA in DEPC water

Quality control and making the spike mix:

All *in vitro* transcribed RNA was then checked by both agarose gel electrophoresis and the Nanodrop. The RNA concentration was then adjusted so that each spike RNA concentration was approximately 1 µg / µl. The RNA was then aliquotted and stored at -80 °C

Each *Arabidopsis* RNA was then mixed and this mixture is spiked into each reverse transcription and labelling reaction performed by FlyChip.

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