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Product Information

***In Vitro* Director™ PCR System – 5' FLAG®**

Catalog Number **ID0030**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Sigma's *In Vitro* Director™ PCR System – 5' FLAG kit provides all reagents needed for the rapid generation of high quality, expression-ready DNA templates that can be used with *in vitro* transcription/translation systems to produce N-terminal FLAG-tagged fusion proteins. *In vitro* synthesized fusion proteins are very useful for rapid analysis of gene functions, such as enzymatic activity, protein-protein interaction, function domain mapping, and structure analysis.

The PCR system uses the Director method, a novel method that facilitates the directional ligation of transcription/translation regulatory sequences (Anchors™) to the PCR products. It differs from conventional PCR kits in that it uses a specially formulated dNTP mix containing dATPαS and dGTPαS. After PCR, the cohesive 5' termini of the amplicon are produced by Exonuclease III digestion instead of being generated by traditional restriction enzyme digests. Incorporation of dA/GTPαS into the amplicon protects the amplicon from overdigestion by Exonuclease III.[1]

The kit contains paired 5' and 3' Anchors as well as universal primers complementary to the Anchor sequences. The 5' FLAG Anchor is a double-stranded DNA containing the T7 promoter, Kozak sequence, FLAG sequence, and a *Hin*d III overhang. The 3' Anchor is also a double-stranded DNA containing a *Bgl* II overhang and terminator sequence.

The universal process of rapidly generating expression-ready DNA templates involves five steps: a first round PCR using gene-specific primers, Exonuclease III digestion, PCR clean-up, ligation of the PCR amplicon to the 5' and 3' Anchors, and a second round of PCR using Anchor primers.

Reagents

The kit contains sufficient reagents to perform 25 reactions.

Kit Components	Product Number	Quantity
JumpStart™ REDAccu*Taq*™ LA DNA Polymerase	D 1938	2 vials (125 µl)
10x Accu*Taq*™ LA DNA Polymerase Buffer	B 0174	0.5 ml
ExoClone™ dNTP Mix (20x)	E 4280	1 vial (62.5 µl)
Control PCR Template	D 4314	10 µl (1 ng/µl)
Control RDC Primer-F	P 4986	25 µl (20 µM)
Control RDC Primer-R	P 5111	25 µl (20 µM)
Exonuclease III	E 1131	25 µl (100 U/µl)
dNTP Mix, 10 mM	D 7295	0.2 ml
Water Molecular Biology Reagent	W 4502	1.5 ml
5' FLAG Anchor	F 0179	50 µl (1 ng/µl)
3' Anchor	A 7228	50 µl (1 ng/µl)
Anchor Primer- Forward	A 7478	25 µl (20 µM)
Anchor Primer-Reverse	A 7353	25 µl (20 µM)

Reagents and Equipment Required but not Provided

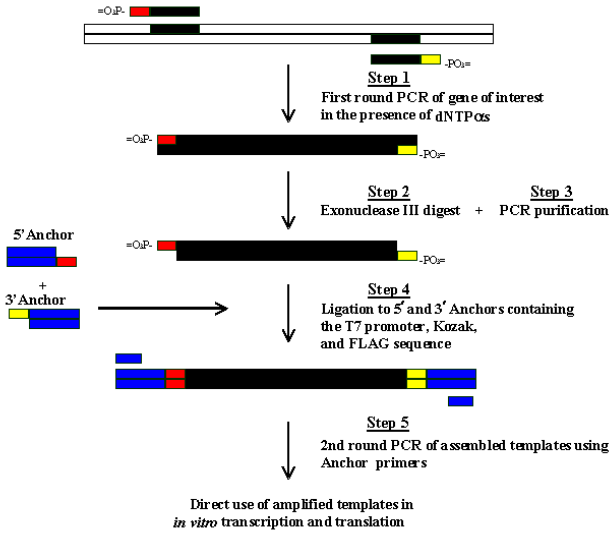
- Template DNA and 5' phosphorylated gene-specific primers with *Hin*d III/*Bgl* II overhangs
- Pipettes and aerosol resistant pipette tips
- 0.5 or 0.2 ml thin-walled PCR micro-centrifuge tubes
- Thermal cycler
- GenElute™ PCR Clean-up Kit (Product Code NA1020) or equivalent
- T4 DNA Ligase with 10x Buffer (Product Code D 1567)

In vitro TNT® Wheat Germ Extract System (Product Code TN0100) or TNT T7 Quick for PCR DNA rabbit reticulocyte system (Promega).

Precautions and Disclaimer
This kit is for laboratory use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
All components should be stored at –20 °C. Avoid multiple freeze-thaw cycles.

Procedure
Outline



General Considerations
Template DNA
The kit is compatible with different sources of DNA template, including plasmid DNA, cDNA, and genomic DNA. However, PCR performance may be affected by the complexity and purity of the DNA template.

Primer Design
The gene specific primers require 5' phosphorylation and *Hin*d III/*Bgl* II overhangs. The 5' forward primer requires the addition of 5 base pairs corresponding to the *Hin*d III overhang (5'-P-AGCTT - target sequence – 3'). The 3' Reverse primer requires the addition of 5 base pairs corresponding to the *Bgl* II overhang (5'-P-GATCT - target sequence – 3'). Primers should be ≥20 bp (specific target sequence plus 5 bases for overhangs) to achieve adequate specificity; however, the length can vary depending on the sequence content of the selected region. Phosphorylation is necessary for efficient ligation and can be done during primer synthesis. The T_m, GC content of primers, and complexity of the template DNA are important considerations in successful PCR. We recommend the T_m of primers be between 65-75 °C with the GC content between 40-60%. The T_m difference of forward and reverse primers should not exceed ±5 °C.

DNA Polymerases
The kit uses JumpStart REDAccu*Taq* LA DNA polymerase for PCR amplification. This special enzyme blend offers several advantages over conventional *Taq* polymerases: excellent amplification for long-distance PCR (up to 22 kb for complex genomic DNA and 40 kb for less-complex templates); high fidelity (6.5x higher than standard *Taq* polymerase and comparable to *Pfu* polymerase); hot-start mechanism using JumpStart *Taq* antibody to prevent nonspecific product formation, improving specificity; and direct loading of PCR products onto an agarose gel without the addition of loading buffers, saving time in product characterization. Other DNA polymerases are also compatible with this kit; however, additional PCR optimization may be required to obtain comparable results.

PCR Conditions
PCR conditions need to be determined empirically for each set of specific primers and template. It is critical to optimize PCR conditions for high specificity, i.e., generating a single product band, to avoid the need for further gel purification. A "hot-start" mechanism using JumpStart *Taq* antibody, which significantly improves PCR specificity, has been incorporated in this kit. For most applications, you should only need to adjust the annealing temperature and extension time according to the T_m of the primers and the length of the template. However, other techniques such as using "touchdown" PCR may further improve the specificity of the amplification.[3]

Step 1. First Round PCR
Amplify the gene of interest using phosphorylated gene specific primers (5' forward primer = 5'-P-AGCTT - target sequence, 3' Reverse primer = 5'-P-GATCT - target sequence) and PCR system. Mix the following components in a 0.2 ml or a 0.5 ml PCR tube on ice.

	Control reaction:
5 µl	10x Accu*Taq* LA DNA Polymerase Buffer
2.5 µl	ExoClone dNTP mix (20x) (teal cap)
1 µl	Control RDC Primer-F (20 µM)
1 µl	Control RDC Primer-R (20 µM)
1 µl	Control PCR template (1 ng/µl)
2.5 µl	JumpStart REDAccu*Taq* LA DNA Polymerase
37 µl	Water
50 µl	Total volume

Sample reaction:

5 µl	10x Accu*Taq* LA DNA Polymerase Buffer
2.5 µl	ExoClone dNTP mix (20x) (teal cap)
1 µl	Gene specific primer-forward (20 µM)
1 µl	Gene specific primer-reverse (20 µM)
x µl	Target sequence template*
2.5 µl	JumpStart REDAccu*Taq* LA DNA Polymerase
38-*x* µl	Water
50 µl	Total volume

*Note: if using simple template such as plasmid, 1 ng target template should be sufficient; if using complex template, such as cDNA mixture, 50 – 500 ng template is needed.

Control PCR:

Initial denaturation:	95 °C, 5 minutes
30 cycles:	
denaturation	94 °C, 30 seconds
annealing	62 °C, 30 seconds
extension	68 °C, 2 minutes
Final extension:	68 °C, 7 minutes
Soak:	4 °C

Sample PCR:

The PCR amplification profile will vary depending on the T_m value of the specific primer set and the length of the template. We recommend setting the annealing temperature 3-5 °C below the T_m of the gene specific primers and the extension time at 1-1.5 minute per kb of target length. If the T_m of the gene specific primers is between 65-72 °C, the control conditions can be used. However, if the T_m of the gene specific primers is above 75 °C, use a two-step PCR profile (94 °C for denaturation and 68 °C or 72 °C for annealing/extension).

Step 2. Exonuclease III digestion

After PCR, add 1 µl of Exonuclease III to each 50 µl PCR (final concentration of 2 U/µl). Mix thoroughly and incubate at 37 °C for 10 minutes. After the digestion remove a 3-5 µl aliquot of each reaction for agarose gel analysis.

Step 3. Purification of PCR products

If PCR results reveal a single amplification band with a reasonable yield, proceed with a post-reaction clean-up using GenElute PCR Clean-up Kit or equivalent system.

If PCR results reveal more than one band, gel extraction and purification should be performed in order to obtain a specific PCR product. The GenElute Gel Extraction kit or equivalent system may be used.

If no PCR product is observed, check the PCR conditions. Refer to the troubleshooting guide in this technical bulletin for assistance with optimizing PCR conditions.

Step 4. Ligation

Ligate Anchors to the amplified gene. Typically, 1-2 µl of amplified gene product is all that is necessary in the ligation reaction. However, longer genes (> 1 kb) may require increasing the amount added to the ligation reaction to ensure adequate ligation efficiency.

Ligation reaction:

14-*x* µl	Water
x µl	first round PCR product (20 – 100 ng)
2 µl	5' FLAG Anchor (1 ng/µl)
2 µl	3' Anchor (1 ng/µl)
2 µl	10x T4 DNA Ligase buffer
1 µl	T4 DNA ligase
21 µl	Total volume

Mix thoroughly and incubate at room temperature for 30 minutes.

Step 5. Second Round PCR

Perform second round amplification of assembled template using Anchor primers. Mix the following components in a 0.2 ml or a 0.5 ml PCR tube on ice.

Control and sample reaction:

2 µl	ligation reaction
1 µl	Anchor primer-Forward (20 µM)
1 µl	Anchor primer-Reverse (20 µM)
2.5 µl	JumpStart REDAccu*Taq* LA DNA Polymerase
5 µl	Accu*Taq* LA DNA Polymerase Buffer
2.5 µl	dNTP mix, 10 mM (yellow cap)
36 µl	Water
50 µl	Total volume

Control and sample PCR:

Initial denaturation:	95 °C, 5 minutes
35 cycles:	
denaturation	94 °C, 30 seconds
annealing	60 °C, 30 seconds
extension	68 °C, 2 minutes 30 seconds
Final extension:	68 °C, 10 minutes
Soak:	4 °C

Note: If the assembled template is >2 kb, it may be necessary to modify the PCR profile with a longer extension time.

Remove a 3-5 µl aliquot of each reaction to check on an agarose gel. Refer to the troubleshooting guide in this technical bulletin if a single band is not visible.

Step 6. Purification (optional) and Use of Expression-Ready Template

Samples may be purified before use in *in vitro* transcription/translation reactions using the GenElute PCR Clean-up Kit or equivalent system. This step is optional for some transcription/ translation kits. TNT T7 Quick for PCR DNA does not require purification. However, purification is recommended for use in the TNT T7 Coupled Wheat Germ Extract System.

Templates are ready for use in any *in vitro* T7 transcription system or any coupled transcription/translation system for eukaryotic expression. Please refer to the manufacturer's specific instructions regarding use of PCR products in the particular transcription/translation system.

Related Products for Downstream Analysis

The *in vitro* produced N-terminal FLAG fusion protein may be analyzed utilizing a variety of FLAG and ANTI-FLAG® antibodies, resins, and affinity capture plates. The gels (resins) or plates are useful in purification of the products from proteins in the expression system and simplify downstream analysis. In addition, these platforms provide convenient methods for assays such as protein-protein interaction and function domain mapping. For a complete listing of FLAG and ANTI-FLAG products, please visit www.sigma-aldrich.com.

- ANTI-FLAG M2 Monoclonal Antibody, unconjugated (Product Code F 3165)
- ANTI-FLAG M2-Peroxidase (HRP) conjugate (Product Code A 8592)
- ANTI-FLAG M2-Agarose Affinity Gel (Product Code A 2220)
- ANTI-FLAG HS M2 Coated 96 well plates (Product Code P 2983)
- FLAG 96 well immunoprecipitation kit (Product Code HT COIP-1)

References

1. Putney, S. D., et al., A DNA fragment with an alpha-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated *in vivo.* Proc. Natl. Acad. Sci. USA **78**, 7350-7354 (1981).

2. Olsen, D. B, and Eckstein, F., Incomplete primer extension during in vitro DNA amplification catalyzed by *Taq* polymerase; exploitation for DNA sequencing. Nucleic Acids Res. **17**, 9613-9620 (1989).

3. Don, R. H., et al. "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19**, 4008 (1991).

Troubleshooting guide

Problem	Possible cause	Solution
A first round PCR product is not observed	A PCR component may be missing or degraded	A positive control should always be run to ensure components are functioning.
	Too few PCR cycles performed	Increase the number of cycles in 3-5 cycle increments
	Annealing temperature may be too high	Decrease the annealing temperature in 2-4 °C increments
	Primer design may not be optimal	Confirm the accuracy of the sequence information. If the primer is less than 15 nucleotides long, try to lengthen the primer to 20 nucleotides (in addition to the 5 base pairs added to the 5' end)
	Not enough DNA template	If increasing the number of cycles has no effect, repeat the reaction with a higher concentration of template
	Poor quality DNA template	Evaluate the template integrity by agarose gel electrophoresis. It may be necessary to repurify the template using methods that minimize shearing and nicking
	Denaturation temperature may be too high or too low	Optimize the denaturation temperature by raising or lowering it in 1 °C increments
	Extension time may be too short	Increase the extension time in 1 minute increments, especially for long templates
	Mg^{++}levels may be too low	Optimize the magnesium concentration by using a PCR optimization kit (Product Code M1932)
Multiple PCR products are observed	Too many PCR cycles performed	By reducing the number of cycles, nonspecific bands may be eliminated
	Primer design may not be optimal	Confirm the accuracy of the sequence information. If the primer is less than 15 nucleotides long, try to lengthen the primer to 20 nucleotides (in addition to the 5 base pairs added to the 5' end)
	Annealing temperature may be too low	Increase the annealing/extension temperature in 2-3 °C increments
A second Round PCR product is not observed	A ligation reaction component may be missing or degraded	A positive control should be run to ensure components are functional
	The PCR product may contain impurities	Repurify the PCR product
	Exonuclease III incubation time may be too short	Increase the exonuclease III digestion to 30 minutes
	Ligation time may be too short	Increase the ligation time up to 4 hours