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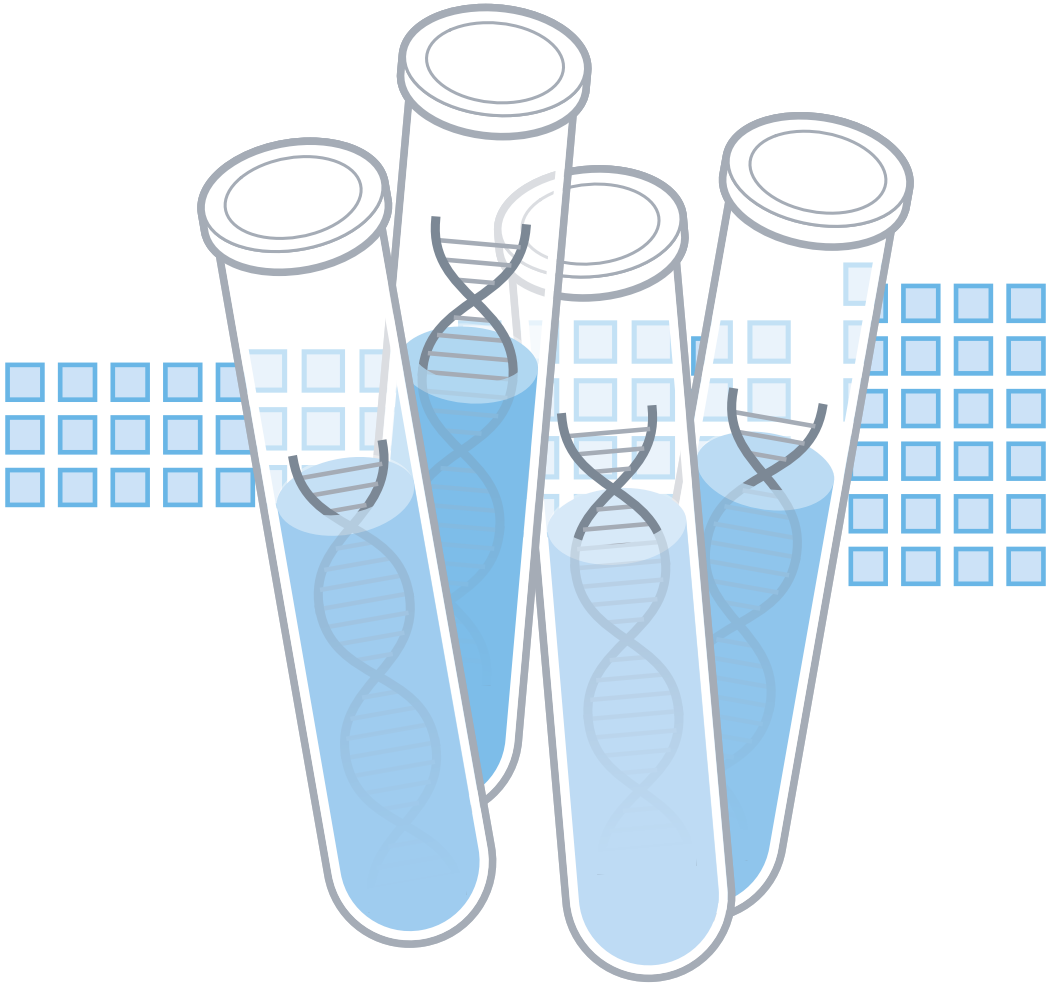
eGene™ Prep Kit User Guide

NC3009: Solubility Tag Screen



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eGene Prep Kit

General information

The eGene™ Prep Kit NC3009 is designed to rapidly convert your synthesized DNA fragments into linear expression constructs that are immediately compatible with the eProtein Discovery™ system.

The kit has been designed for synthesized DNA fragments up to 3000 bp, and we recommend using gBlocks™ from our third party DNA vendor IDT.

In order to proceed with eGene preparation, which is outlined in this manual, you will need to have your synthesized gene fragments in hand.

When focused on obtaining certain target/s, the Solubility Tag Screen allows you to explore a combination of POI variations & solubility tags to increase your chance of obtaining a soluble, active protein (Figure 1).

- Provides robust screen of constructs with different solubility tag options to increase chances of obtaining soluble protein
- 3, 4 or 6 proteins
- 4, 6 or 8 solubility tag options

Figure 1. Possible combinations of POI variations & solubility tags.

The eGene constructs are assembled based on a one pot, one step overlap extension PCR that primarily requires four key components: left megaprimer, right megaprimer, universal terminal primer pair and the DNA sequence of interest corresponding to the target protein of interest (POI) as shown in Figure 2.

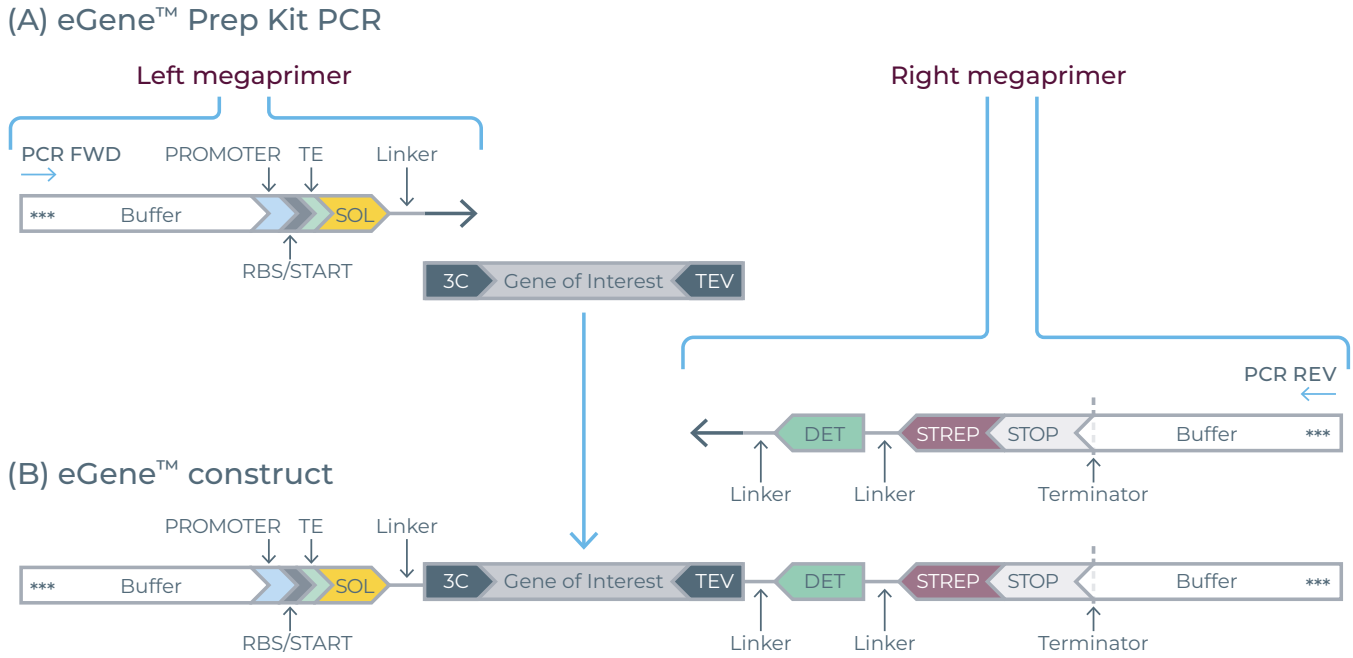


Figure 2. Description of the eGene components including megaprimers and resulting eGene construct. Ribosome binding site (RBS), Translation enhancer (TE), Solubility tag (SOL), 3C and TEV (protease binding sites), Detection tag (DET), streptactin based purification tag (STREP). The terminal primers used in the assembly process contain three phosphorothioate bonds (***) at their 5' ends.

The megaprimers are double stranded DNA molecules containing all the regulatory elements required for transcription and translation.

- The left megaprimer may contain a variety of solubility tags, while the right megaprimer always includes a GFP based detection tag (DET, 17 amino acids long, 1.95 kDa) along with a streptactin based purification tag (STREP).

- Importantly, the left megaprimer incorporates a 3C protease cleavage site at its 3' terminus while the right megaprimer incorporates TEV protease site at the 5' terminus providing the user flexibility to easily cleave off the additional tags from the purified proteins.

- The 3C-TEV protease cleavage sites also serve as the hybridization site in the overlap extension assembly reaction. Therefore, your gene of interest must be pre-adapted with 3C and TEV adaptor sequences at the 5' and 3' ends respectively, to be compatible with the eGene Prep Kit workflow.

During the overlap extension PCR, in the first 5-10 cycles multiple copies of the fully assembled products are formed. Thereafter, the universal primers take over to exponentially amplify the full-length assembled product.

Features and benefits

- **Efficiency** – expand one synthesized DNA fragment into up to eight different expression eGene constructs with different solubility tag options as shown in Table 1
- **Robustness** – individual constructs can yield 60 µL of up to 0.4 pmol DNA following eGene preparation
- **Reliability** – over 800 GOIs have been successfully converted into Grade 1 eGene constructs at the first attempt
- **Speed** – experiment set-up takes less than 30 minutes
- **Accessibility** – eluted, purified DNA is immediately ready-to-use in cell-free protein synthesis reactions

Contents

The eGene Prep kit - Solubility Tag Screen - NC3009 comes with the components listed in Table 1 and shown in Figure 3.

The components are supplied in a Nuclera branded box with a green stripe on the label, and must be used within 18 months from the manufacturing date indicated on the kit box.

Component	Volume	Cap color	Storage temperature	Tube reference number
P17-Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-01
CUSF-Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-02
FH8-Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-03
TRX-Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-04
ZZ-Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-05
SUMO-Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-06
SNUT-Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-07
Strep Primer Mix	135 µL	Blue	-80°C or -20°C	EG2-08
Re-amp Primer Mix	200 µL	Yellow	-80°C or -20°C	EG2-10
10x Control Template	10 µL	Purple	-80°C or -20°C	EG2-11
Elution Buffer	18 mL	White	-80°C *	EG2-09

Table 1. List of components in the eGene Prep kit - Solubility Tag Screen - NC3009.

* at first use, make small 1.5 mL aliquots of the Elution Buffer, freeze at -80°C and treat as single use.

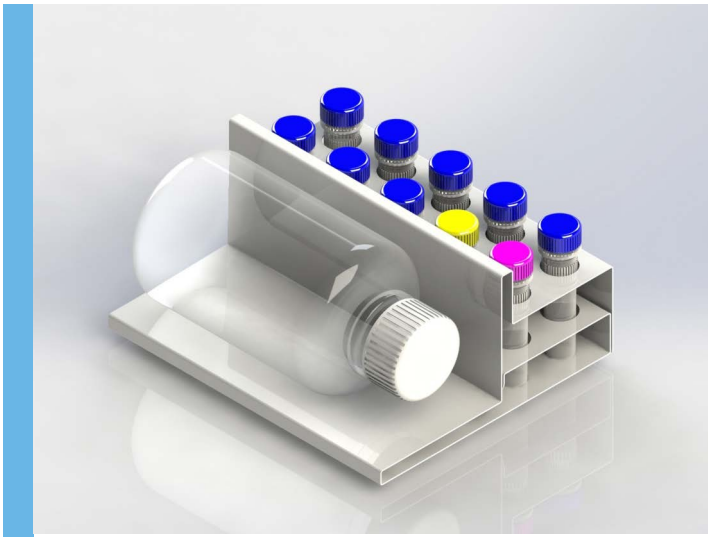


Figure 3. Components in the eGene Prep Kit - Solubility Tag Screen - NC3009.

Contents description

▹ **Primer Mix** formulations containing optimized concentrations of megaprimers corresponding to the left and right construct flanks, and universal forward and reverse terminal primers.

The eight megaprimer formulations allow the preparation of eight different linear expression constructs with or without varying N-terminal solubility tags (Table 2).

Component	N-term SOL	C-term DET	C-term PUR	eGene construct
P17-Strep Primer Mix	P17	Yes	Yes	P17-/POI/-DET-STREP
CUSF-Strep Primer Mix	CUSF	Yes	Yes	CUSF-/POI/-DET-STREP
FH8-Strep Primer Mix	FH8	Yes	Yes	FH8-/POI/-DET-STREP
TRX-Strep Primer Mix	TRX	Yes	Yes	TRX-/POI/-DET-STREP
ZZ-Strep Primer Mix	ZZ	Yes	Yes	ZZ-/POI/-DET-STREP
SUMO-Strep Primer Mix	SUMO	Yes	Yes	SUMO-/POI/-DET-STREP
SNUT-Strep Primer Mix	SNUT	Yes	Yes	SNUT-/POI/-DET-STREP
Strep Primer Mix		Yes	Yes	-/POI/-DET-STREP

Table 2. Primer Mix components included in eGene Prep Kit NC3009.

▹ **Elution Buffer** required for the eProtein Discovery platform.

Note: Before first use, leave the Elution Buffer to thaw on ice for about 2 hours. Once open, make small 1.5 mL aliquots, store at -80°C and treat as single use.

▹ **Re-amp Primer Mix** for troubleshooting, or to produce larger quantities of DNA to scale up protein production (optional).

▹ **10x Control Template**, supplied as a PCR positive control to verify that the prepared formulation and thermocycling parameters are correct.

User supplied reagents / components (not included in the kit)

▹ Synthesised DNA fragments corresponding to the genes of interest (GOI) pre-adapted with 3C and TEV sequences on 5' and 3' ends, respectively. The GOI can be from 125 to 2955 bp long. We recommend gBlocks™ by IDT designed using the eProtein Discovery software.
▹ High fidelity PCR mastermix recommended
▹ Nuclease free water (NFW)
▹ PCR purification kit (column- or bead-based methods)
▹ 1% (w/v) agarose gel
▹ DNA gel stain
▹ Loading buffer
▹ Electrophoresis running buffer
▹ DNA ladder

User supplied equipment

- Thermocycler
- Electrophoresis apparatus
- Gel doc or transilluminator
- Standard benchtop microcentrifuge
- Pipettes with disposable filter tips
- 0.2 mL thin-walled PCR tubes or 96-well PCR plate

eGene preparation workflow

Important notes to convert your synthesized DNA fragments into linear expression constructs successfully:

1. During eGene DNA construct (prep or re-synthesis) purification, eGene Elution Buffer must be used to ensure compatibility with eProtein Discovery Cartridges. Sub-dilutions must also be performed using the eGene Elution Buffer.

2. We recommend using filter tips throughout the protocol to prevent cross-contamination.

3. When outsourcing linear DNA fragments, the length of the DNA of interest (excluding Nuclera adaptor sequences) should be between 125 and 2955 base pairs.

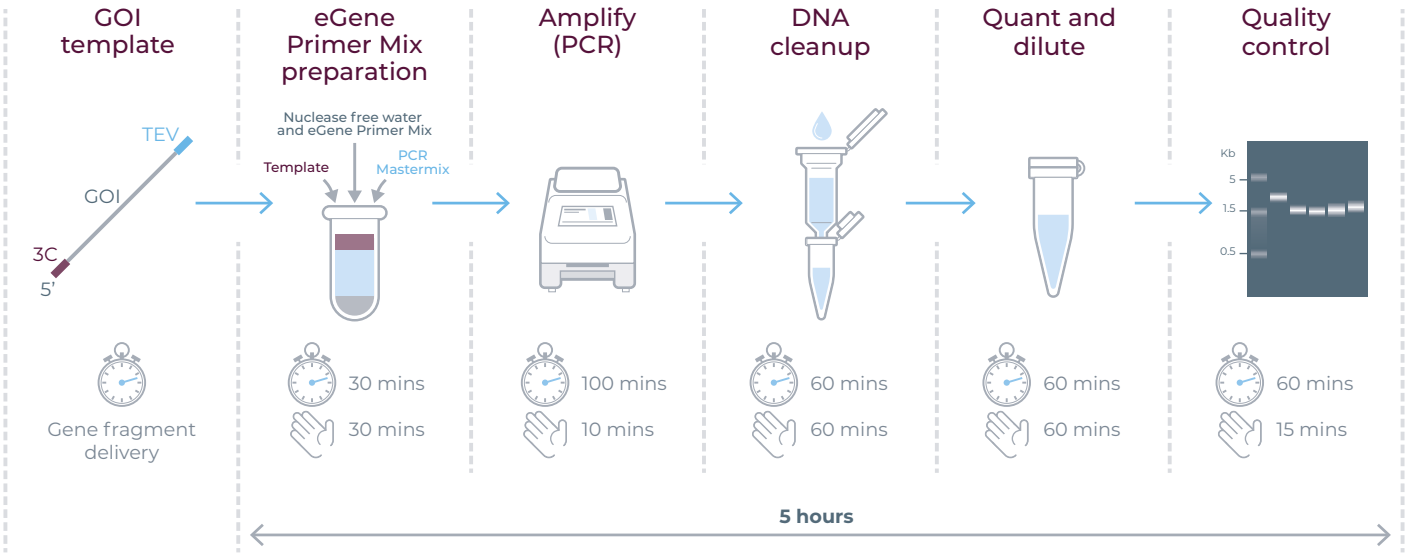


Figure 4. eGene workflow summary - timings applicable for generating eGene constructs using the eGene Prep Kit. For each step of the workflow, the clock indicates the total experiment time and the hand indicates the total hands-on time.

Preparation of the template DNA

Note: If template DNA are gene fragments (gBlock™) supplied by IDT, follow the following steps to dilute it:

1. Before opening the tube, spin it down in a microcentrifuge for 5 seconds to ensure all the lyophilized DNA fragment from IDT is at the bottom of the tube.

   The lyophilized DNA fragment pellet can be statically charged and adhere to the tube wall or cap, resulting in loss of material.

2. Add a suitable buffer such as 0.1X TE, to reach a final concentration of 10 ng/µL. For example, if the tube has 1000 ng of lyophilized product, add 100 µL of molecular grade water, or a buffer to resuspend it.

3. Vortex briefly to resuspend DNA fragments into solution.

4. Either leave the solution at +4°C overnight or incubate at 50°C for 15–20 min. Both methods will ensure that the entire pellet will be resuspended in the buffer.

5. Briefly vortex and centrifuge.

6. Convert DNA concentration to molarity using the following formula:

$$GOI\ (nM) = \frac{Concentration\ (ng\ /\ \mu L)}{[\ Length\ (bp) \times 617.96\ g\ /\ mol\ /\ bp\ ] + 36.04\ g\ /\ mol} \times 1{,}000{,}000$$

Preparation of the template solutions

1. **PCR control template:** In a 200 µL thin-walled PCR tube, add 1 µl of 10x Control Template to 9 µL of nuclease-free water to obtain a Control Template solution at 2 nM and keep on ice.

2. **Gene of interest template:** Prepare a fresh 10 µL solution of the GOI template normalized to 2 nM using the following formula and keep on ice.

$$Volume\ eGene\ (\mu L) = \frac{2\ (Target\ Molar\ Concentration\ in\ nM)\ X\ 10\ (eGene\ Elution\ Buffer\ in\ \mu L)}{Obtained\ Molar\ Concentration\ (nM)\ -\ 2\ (Target\ Molar\ Concentration\ in\ nM)}$$

   Example: For a 2159 bp gene of interest obtained at 10 ng/µL, which corresponds to 7.5 nM, add 3.6 µL of DNA template to 10 µL of eGene Elution Buffer.

Preparation of the PCR reaction mix

The assembly reaction consists of adding the template DNA to the reaction mix containing a Primer Mix and a 2x polymerase mastermix (Figure 5).

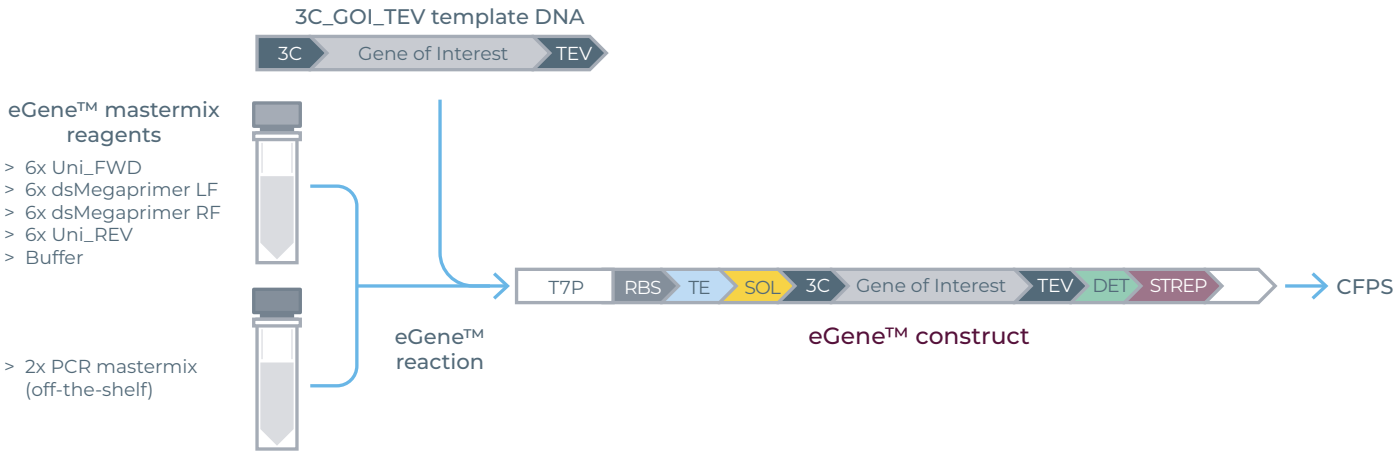


Figure 5. Overview of the eGene Prep Kit assembly reaction set-up. T7 promoter (T7p), Ribosome Binding Site (RBS), translation enhancer (TE), solubility tag (SOL), 3C (human rhinovirus) protease cleavage site (3C), TEV (Tobacco etch virus) protease cleavage site (TEV), detection tag (DET), Strep purification tag (STREP).

1. Thaw on ice each of the individual vials containing the Primer Mixes (see details in Table 2). After use, refreeze any unused material.

   The number of reactions varies depending on the experimental format planned to be applied with the eProtein Discovery instrument:
   - ▷ 3 proteins of interest with 8 eGene constructs for each protein
   - ▷ 4 proteins of interest with 6 eGene constructs for each protein
   - ▷ 6 proteins of interest with 4 eGene constructs for each protein

2. Quick spin down the tube for 10 seconds using a microcentrifuge.

3. To facilitate the work you can prepare a mastermix for each GOI by adding nuclease free water, 2x PCR mastermix and GOI. Aliquot 50 µL of the mastermix into PCR strip tubes/wells followed by addition of 10 µL of the eGene Primer Mix into the respective tubes/wells. Prepare a negative control and a positive control independently.

   Note: Use randomly one of the 8 Primer Mix reagents provided in the kit in the two wells or PCR tubes for the positive and negative controls

Reagent	Reaction volume
Nuclease free water (NFW)	19 µL
2X PCR mastermix (high fidelity PCR mastermix, user supplied)	30 µL
Gene of interest at 2 nM, or water (negative control), or Control Template (positive control)	1 µL
Primer Mix	10 µL
Final volume (maximum recommended volume per reaction)	**60 µL**

Table 3. Preparation of the PCR reactions.

PCR assembly

1. Gently vortex the mixture to produce a homogeneous reaction, then centrifuge briefly to collect the solution at the bottom of the tube.

2. Place your samples in a thermal block cycler and perform PCR using the parameters in Table 4.

PCR step	Temperature	Time	Number of cycles
Pre-incubation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	27
Annealing	60°C	20 seconds	
Elongation	72°C *	30 seconds per kb **	
Final elongation	72°C *	2 minutes	1

Table 4. Thermocycler parameters.

* Elongation temperature: to be based on manufacturer's recommendation.
** Elongation time: to be based on manufacturer's recommendation but should not be less than 30 seconds per kb.

Evaluation of the amplified DNA by agarose gel electrophoresis

1. Prepare a 1% (w/v) agarose gel with a DNA gel stain in 1x Tris acetate EDTA (TAE) or 1x Tris-borate-EDTA (TBE) buffer.

2. Using new wells or new PCR tubes, prepare 12 µL samples for loading on the gel as detailed in Table 5.

6x gel loading dye	Nuclease free water	eGene DNA construct
2 µL	8 µL	2µL

Table 5. Sample preparation for agarose gel electrophoresis.

Note: a bulk solution of ready-to-use 1x loading buffer can be made and stored at room temperature or +4°C.

3. Load 10 µL of PCR products and control on 1% agarose gel along with an appropriate DNA ladder (1 kb).

If the purity of the eGene constructs is low with the presence of one or more extra band(s) on the gel, there is a risk to obtain the protein of interest with a significant amount of impurities.

Troubleshooting: For eGene constructs containing more than one band, a band-stab and reamplification is required using the Re-amp Primer Mix provided in the eGene Prep kit. The band-stab and reamplification protocols can be found in the Troubleshooting section of this document.

PCR purification

The PCR reactions must be cleaned using the DNA purification method of your choice, either column or bead-based methods. Gel extraction is not recommended as it might result in significant product yield loss.

Important note: The samples must be eluted using the Elution Buffer (50 µL) supplied in the eGene Prep kit to make them compatible with the eProtein Discovery cartridge. The eGene Elution Buffer is a HEPES buffer, pH 8.0, containing a surfactant.

DNA quantification

1. DNA quantification (ng/µL) can be carried out using spectrophotometric or fluorescent dye-based methods.

   Blanks must be done using the Elution Buffer provided in the eGene Prep kit.

2. Determine the length of the eGene using the calculator available on the eProtein Discovery Software or the formula below and Table 6.

   Note: the length of the GOI should not include the length of the 3C and TEV sequences.
   Note: the length of the Control Template supplied in the kit is 660 bp, 3C and TEV sequence lengths not included.

   eGene Length (bp) = GOI Length (bp) + Primer Mix Length (bp)

Primer Mix	Total length of the flanks (bp)
P17-Strep	1134
CUSF-Strep	1302
FH8-Strep	1239
TRX-Strep	1359
ZZ-Strep	1383
SUMO-Strep	1341
SNUT-Strep	1476
-Strep only	990

Table 6. Total lengths of the N-terminal and C-terminal flanks to add to the gene of interest to determine the total length of the eGene constructs

3. Calculate the molar concentration (nM) of the eGene constructs using the calculator available on the eProtein Discovery Software or the following equation:

$$GOI\ (nM) = \frac{Concentration\ (ng\ /\ \mu L)}{[\ Length\ (bp)\ x\ 617.96\ g\ /\ mol\ /\ bp\ ] + 36.04\ g\ /\ mol} \text{ x } 1{,}000{,}000$$

   Example: the molar concentration of a 1415 bp long eGene at 60 ng/µL is 68.6 nM.

$$\frac{60}{(1415\ x\ 617.96) + 36.04} \text{ x } 1{,}000{,}000 = 68.6\ nM$$

   Note: If the DNA concentration is lower than 5 nM, please refer to the reamplification protocol in the troubleshooting section to generate more DNA.

eGene constructs normalization

To be compatible with the eProtein Discovery platform, all eGene constructs must be normalized to 5 nM using the Elution Buffer provided in the eGene Prep Kit .

60 µL of an eGene at 5 nM is sufficient for one experimental run on the eProtein Discovery instrument and a 200 µL scale-up experiment.

Determine the volume of eGene constructs to add to 60 µL of eGene Elution Buffer using the following formula:

$$\textit{Volume eGene (µL)} = \frac{\textit{5 (Target Molar Concentration in nM) X 60 (eGene Elution Buffer in µL)}}{\textit{Obtained Molar Concentration (nM) – 5 (Target Molar Concentration in nM)}}$$

Example: For a 2589 bp eGene obtained at 40 ng/µL, which corresponds to 25 nM, add 15 µL of DNA sample to 60 µL of eGene Elution Buffer.

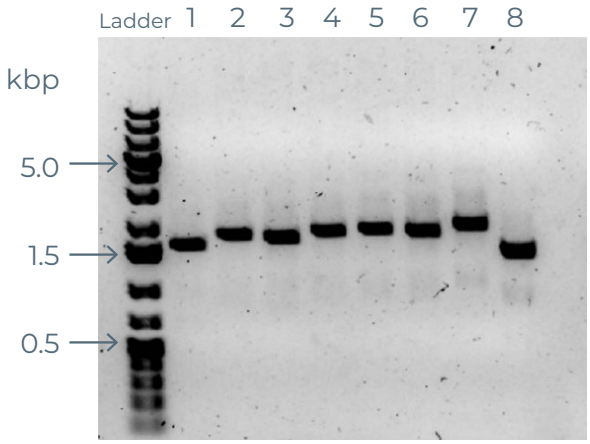
Final gel QC

It is recommended to run 2 µL of the samples on a 1% agarose gel post PCR clean-up.

Note: This step is optional if you already evaluated the amplified DNA by electrophoresis after the PCR assembly.

eGene constructs must be of high purity since the input DNA sequence is transcribed in the Cell-free Protein Synthesis reaction and the subsequent mRNA is translated to the final protein of interest.

The quality of DNA generated using the eGene Prep Kit - Solubility Tag Screen (NC3009) is illustrated in Figure 6 (agarose gel) and 7 (electropherogram), showing a comparison of the PCR products from incorporating different solubility tags using the standard solubility tag screen, while keeping the 'Gene of Interest' (GOI) constant.



Lane	Description	Lane	Description
1	P17-GOI-DET-STREP	5	ZZ-GOI-DET-STREP
2	CUSF-GOI-DET-STREP	6	SUMO-GOI-DET-STREP
3	FH8-GOI-DET-STREP	7	SNUT-GOI-DET-STREP
4	TRX-GOI-DET-STREP	8	GOI-DET-STREP

Figure 6. Example of an agarose gel illustrating the DNA bands generated from PCR products of the GOI CALM1 (UniProt ID P0DP23) expanded with different solubility tags.

Lane	Description	Lane	Description
1	P17-GOI-DET-STREP	5	ZZ-GOI-DET-STREP
2	CUSF-GOI-DET-STREP	6	SUMO-GOI-DET-STREP
3	FH8-GOI-DET-STREP	7	SNUT-GOI-DET-STREP
4	TRX-GOI-DET-STREP	8	GOI-DET-STREP

Figure 7. Example of a capillary electrophoresis generated electropherogram illustrating the DNA bands generated from PCR products of the GOI rpsA (UniProt ID P0AG67) expanded with different solubility tags.

If the purity of the eGene constructs is low with the presence of one or more extra band(s) on the gel, there is a risk to obtain the protein of interest with a significant amount of impurities.

Troubleshooting: For eGene constructs containing more than one band, a band-stab and reamplification is required using the Re-amp Primer Mix provided in the eGene Prep kit. The band-stab and reamplification protocols can be found in the Troubleshooting section of this document.

Storage of the eGene constructs

Purified and normalized eGene constructs can be stored at -20°C short term (days) or -80°C for long term storage.

eGene Optimization / Troubleshooting

1. Band-stab protocol

Note: Always wear UV safety glasses when working with a transilluminator.

Overview: Band-stab is suggested to obtain pure eGene constructs directly from the eGene PCR assembly using the eGene Prep Kit. An example of a band-stabbed gel is in Figure 8.

It is a simple method to obtain single bands as compared to gel purification techniques which requires excision and purification of the DNA fragment from the agarose gel.

The method described in this section is adapted from the publication by Bjourson and Cooper (*Bjourson AJ, Cooper JE. Band-stab PCR: a simple technique for the purification of individual PCR products. Nucleic Acids Res. 1992 Sep 11; 20(17): 4675*).

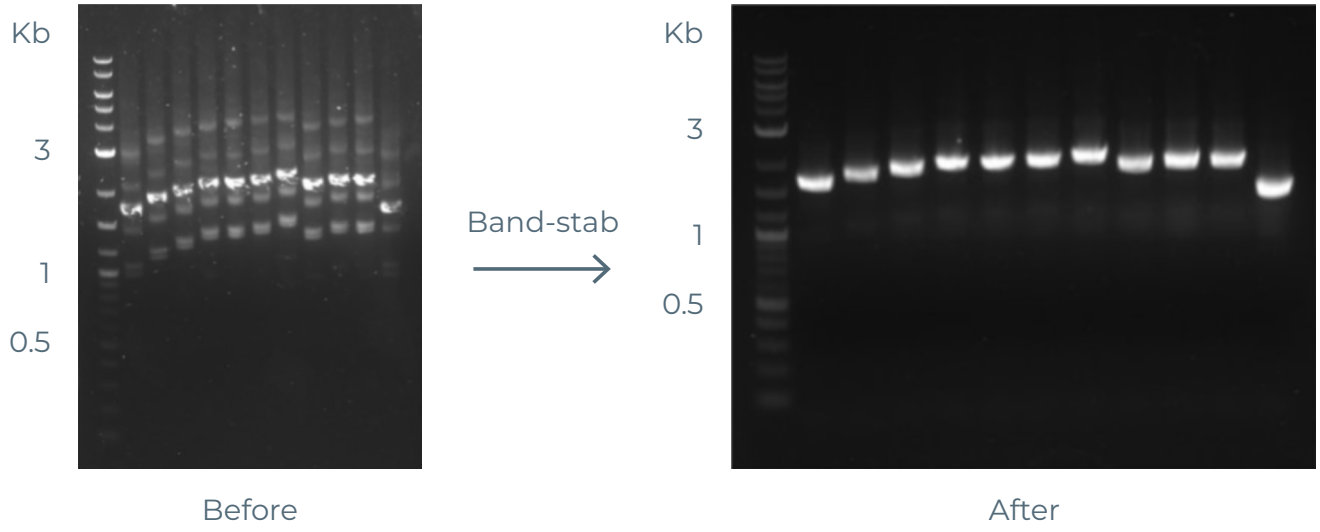


Figure 8. Example of band-stabbed gel.

1. Run the eGene constructs showing non-specific bands on a 1 % (w/v) agarose gel, or use the gel made for the final gel QC step.

2. Load 2 µL of purified product on the gel.

3. Set-up the electrophoresis apparatus for 45 minutes at 120 Volts, or until the desired band is well separated.

4. While the electrophoresis is running, prepare the reamplification PCR reaction as described in Section 2. Reamplification.

5. When the electrophoresis run is complete, take the gel from the tray and take a picture using an imaging apparatus and locate where your desired bands are.

6. Place the gel on the transilluminator and use the blue light setting.

7. Locate your band of interest and take a P10 pipette with a P10 filter tip attached.

8. Hold the pipette vertically on top of the desired band and stab the band three times per PCR reaction.

9. Transfer the pipette tip into the PCR reamplification reaction mix. It is recommended to pipette up and down three times to release all the material from the pipette tip into the PCR reaction solution.

Note: avoid transferring pieces of gel into the PCR reaction mix as they may inhibit the amplification process.

2. Reamplification

General information
The reamplification employs the same principles of a regular PCR reaction.
This method allows synthesizing more of a eGene construct, either collected from a band-stab protocol, or in the case the quantity is not sufficient for a use on the eProtein Discovery instrument or for a scale-up experiment.

Notes:
- Always use an original eGene assembly as template for PCR reamplification. It is not recommended to use a reamplified product as a template as this would increase the risk of mutations over the subsequent generations.

- The minimum number of reactions that should be prepared is 3 x 50 µL:
  - The first reaction is with the template eGene construct
  - The second reaction serves as a no template control.
  - The third reaction is prepared as a spare reaction. Due to the different viscosities and temperatures of the reagents, the pipetting volumes can be inaccurate leading to shortage of reaction solution.

- If re-amplifying more than one construct, it is recommended to prepare a sufficient reaction solution containing all the components except the template that are subsequently added to the respective wells or tubes.

- Reamplification product lengths are 40 bp shorter than original eGene constructs due to the use of a nested primer pair to synthesize them.

Preparation of the eGene template DNA to be reamplified
1. Prepare a 10 µL solution of the eGene template normalized to 2 nM and keep on ice.
   Example: For a 2159 bp gene of interest obtained at 10 ng/µL, which corresponds to 7.5 nM, add 3.6 µL of DNA template to 10 µL of eGene Elution Buffer.

Preparation of the reamplification PCR reaction mix
1. Thaw 2x PCR Mastermix and Re-amp Primer Mix at room temperature and spin down tube for 10 seconds at 1000 g.

2. For each reamplification PCR reaction, add the components listed in Table 7 and keep the tubes or the PCR plate on ice.

Note: An additional well or PCR tube should be used as negative control, using nuclease free water instead of the eGene.

Reagent	Reaction volume
Nuclease free water (NFW)	21.5 µL
2X PCR mastermix (high fidelity PCR mastermix, user supplied)	25 µL
Re-amp Primer Mix	2.5 µL
eGene DNA template, or nuclease free water	1 µL
Final Volume	**50 µL**

Table 7. Preparation of the PCR reaction solutions.

Reamplification PCR reaction

1. Gently vortex the mixture to produce a homogeneous reaction, then centrifuge briefly to collect the solution at the bottom of the tube.
2. Place your samples in a thermal block cycler and perform PCR using the parameters in Table 8.

PCR step	Temperature	Time	Number of cycles
Pre-incubation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	19
Annealing	60°C	20 seconds	
Elongation	72°C *	30 seconds per kb **	
Final elongation	72°C *	2 minutes	1

Table 8. Thermocycler parameters.

* Elongation temperature: to be based on manufacturer's recommendation.
** Elongation time: to be based on manufacturer's recommendation but should not be less than 30 seconds per kb.

3. To evaluate, purify, quantify and normalize the obtained re-amplified eGene constructs, follow the same procedure as from section 'Evaluation of the amplified DNA by agarose gel electrophoresis'.

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