RNA LABELING KIT

ULS™

ULS™ microRNA Labeling Kit

Product code

EA-036 / EA-037 / EA-038

For laboratory use only

Not for diagnostic purposes

Instruction manual

This kit is intended for RESEARCH USE ONLY. IT IS NOT INTENDED FOR DIAGNOSTIC APPLICATIONS and/or COMMERCIAL PURPOSES.

Important

Open the kit immediately and store the components at the temperatures as instructed in the tables in section A, page 7

- Read the entire instruction manual before starting your experiment.
- Do not mix reagents from different kits.
- During the preparation of reagents and throughout the entire procedure please observe the safety regulations issued for laboratories concerning handling of samples.
- Dispose of reagents according to relevant local regulations. Take appropriate safety precautions such as wearing a lab coat, gloves and eye protection.

Limited Product Warranty – Patent Disclaimer

This warranty limits our liability to replacement of this product. No other warranties of any kind expressed or implied, including without limitation, implied warranties or fitness for a particular purpose, are provided by KREATECH. KREATECH shall have no liability for any direct, indirect, consequential, or incidental damages arising out of use, or the inability to use this product.

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A. Assay Materials

I. Components and Storage

The ULS microRNA Labeling Kits contains the following items.

Product code: EA-036

Materials Provided	Quantity	Storage Conditions
Cy5-ULS	25 µL	2-8°C
Cy3-ULS	25 µL	2-8°C
10 x Labeling Buffer	100 µL	2-8°C
KREA*pure* columns	20 pcs	2-8°C
KREA*block*	1.5 mL	-20°C[a]

[a] KREA*block* is shipped at 4°C, store immediately at -20°C upon arrival.

Product codes: EA-037 and EA-038

Materials Provided	Quantity	Storage Conditions
Cy3-ULS (EA-037) or Cy5-ULS (EA-038)	50 µL	2-8°C
10 x Labeling Buffer	100 µL	2-8°C
KREA*pure* columns	20 pcs	2-8°C
KREA*block*	1.5 mL	-20°C[a]

[a] KREA*block* is shipped at 4°C, store immediately at -20°C upon arrival.

B. General Information

I. Background

Over the last few years there has been growing interest in detecting non coding small RNA molecules. These short RNA molecules appear to be responsible for regulating gene expression, for example by triggering mRNA degradation by endonucleases (siRNA) or by binding to the mRNA molecules and blocking translation (miRNAs). Labeling of these small RNA molecules is difficult using the common enzymatic labeling methods, but the ULS technology is ideally suited since it chemically labels all nucleic acids efficiently independent of fragment size. The labeling procedure itself is an easy method that can be completed within 30 minutes.

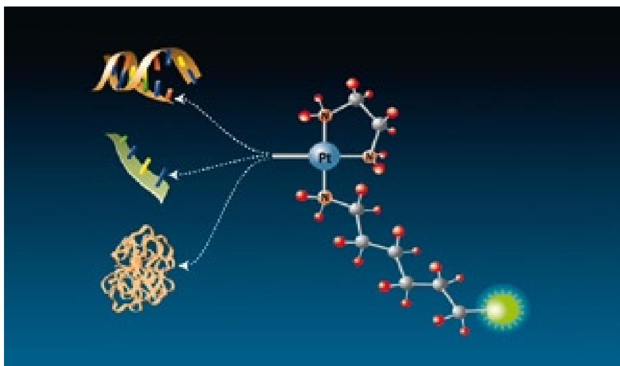
In combination with the ULS labeling system, KREATECH provides two components that play a key role; KREA*pure* and KREA*block*. KREA*pure* columns are specially developed columns that efficiently remove ~99.9% of un-reacted ULS after the labeling reaction and allow fragments of all lengths to pass through unhindered. Recovery using this column is typically above 95%. KREA*block* is a unique blocking solution that helps to reduce background on the microarray.

II. Principle of ULS Labeling

The proprietary ULS technology is based on the stable binding properties of platinum to biomolecules. The ULS molecule consists of a platinum complex, a detectable molecule and a leaving group which is displaced upon reaction with the target. This ULS molecule forms a co-ordinative bond, firmly coupling the ULS to the target. ULS labels DNA and RNA by binding to the N7 position of guanine ULS is available coupled to a variety of fluorochromes and biotin.

ULS thereby enables one-step chemical labeling of nucleic acids within 15-30 minutes. For proteins, ULS binds to nitrogen and sulphur containing side chains of methionine, cysteine and histidine (see figure below).

Schematic overview of the ULS™ technology



Targets

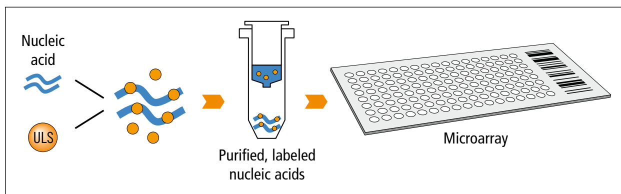
- Nucleic Acids
- Proteins

Coordinative chemistry of the ULS technology

Proteins	Nucleic acids
Methionine, Cysteine, Histidine	Guanine
ULS labels proteins by forming a coordinative bond on the sulfur atoms of methionine, cysteine and the nitrogen atom of histidine.	ULS labels DNA and RNA by forming a coordinative bond on the N7 position of guanine.

III. Overview of Procedure

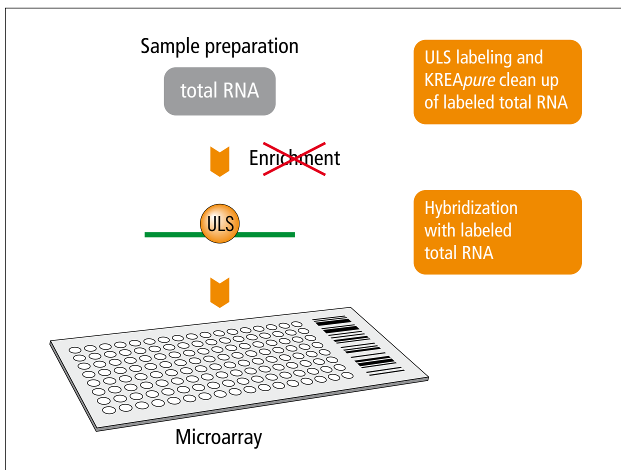
1. Incubate with ULS reagents (15 mins).
2. Removal of non reacted Cy-ULS using KREA*pure* columns
3. Hybridization of the labeled RNA to a microarray.



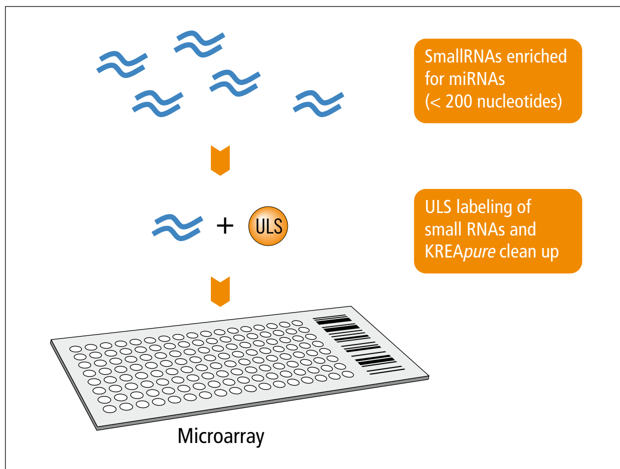
Whole labeling procedure takes less than 30 minutes.

The EA-036/037/038 kits can be used for 2 approaches:

1) Labeling totalRNA directly without an enrichment procedure:



2) Labeling microRNA enriched small RNAs (components for the enrichment are not delivered in the kits):



C. Protocol for using total RNA

Before you begin

See below procedural notes that you should read and understand before starting an experiment.

I. Procedural Notes

For total RNA isolation we recommend to use Trizol (Invitrogen) extraction followed by precipitation or total RNA isolation procedures using the following kits; miRNeasy™ procedure (QIAGEN), *mir*Vana™miRNA Isolation procedure (Ambion/Applied Biosystems), miRACLE™ miRNA isolation kit (Stratagene).

Be aware that some components in silica based purification systems inhibit the ULS labeling reaction. This can be prevented by a final wash step using 80% ethanol (PA) before elution, and elution using ultrapure water instead of elution buffer.

Alternatives: alcohol precipitation of eluted material or cleanup using an EDTA, sephadex combination (if necessary, please contact us for details).

For total RNA OD_{260}/OD_{280} ratio should be >1.90 and OD_{260}/OD_{230} ratio should be >2.10 (see appendix F).
This means that RNA samples need to be clean of divalent cations (eg. Mg^{2+}) salt and other (wash) buffer components that could disturb the ULS labeling efficiency.
When quality criteria are not met do not proceed but contact techservices@kreatech.com.

Make sure that total RNA is free of DNA and protein contamination and is solved in one following buffers compatible with ULS labeling:

- ultrapure water
- TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 or pH 8)
- 10 mM Lithium Chloride
- 10 to 100 mM Sodium acetate
- 10 mM Sodium Chloride

To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves, and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.

Maintain a clean work area.

Avoid repeated freeze-thaw cycles of the KREA*block* component.

II. ULS labeling

1. Determine the concentration of the total RNA by absorbance measurement at 260 nm. Also measure at 230 and 280 nm to determine the purity of the total RNA sample (see Appendix and Procedural Notes for the quality criteria).
2. Determine the RNA integrity by 1% agarose gel electrophoresis or using a Bioanalyzer (Agilent) (for human total RNA, two discrete bands of 28S [5kb] and the 18S [2kb] should be visible, the 28S band being 2-fold more intense).
3. When the total RNA meets the quality criteria, set up labeling reactions as follows (see tabular examples as well).

Note: *the procedure described below is assuming the need to hybridize 1 µg of total RNA per sample on a microarray. In case your application needs more sample to be hybridized, you can scale up accordingly: use a ratio of exactly 1.0 µL of ULS per 1.0 µg of RNA. The optimal RNA concentration for ULS labeling is ≥ 50 ng/µL. If less than 1 µg RNA is available use lower labeling volumes in order to aim for a RNA concentration of 50 ng/µL (e.g. if only 250 ng RNA is available the optimal labeling volume is 5 µL).*

The reagents included in the kit allow you to use up to 2.5 µg of RNA per labeling reaction.

a. Pre-heat a waterbath OR thermocycler at 85°C.
b. Mark RNAse-free PCR tubes or 1.5 mL microcentrifuge tubes to identify samples. Use one tube per samples..
c. Add 1 µg of RNA and ultrapure water to the tube to a total volume of 17.0 µL
d. Add 2.0 µL of 10 x Labeling Solution
e. Add 1.0 µL of Cy3-ULS or Cy5-ULS
f. Mix by pipetting

Example of total RNA labeling using 1 µg of RNA.

	Sample 1	Sample 2
total RNA	X µL (= 1.0 µg)	Y µL (= 1.0 µg)
Cy3-ULS	1 µL	-
Cy5-ULS	-	1.0 µL
10 x Labeling Solution	2.0 µL	2.0 µL
ultrapure water	17.0 - X µL	17.0 - Y µL
Total	20 µL	20 µL

Example of total RNA labeling using 2.5 µg of RNA.

	Sample A	Sample B
total RNA	Q µL (= 2.5 µg)	P µL (=2.5 µg)
Cy3-ULS	2.5 µL	-
Cy5-ULS	-	2.5 µL
10 x Labeling Solution	2.0 µL	2.0 µL
ultrapure water	15.5 - Q µL	15.5 - P µL
Total	20 µL	20 µL

4. Incubate for 15 minutes at 85°C in a PCR machine with hot lid (or a pre-warmed water bath).
5. During the final 10 minutes of this incubation, preparation of the KREA*pure* column can be done (see section III below).
6. Put the sample on ice for at least 1 minute.

   Optional: *If lower labeling volumes are used adjust volume to 20 µL using ultrapure water.*

III. Removal of non-reacted Cy-ULS using KREA*pure* columns

1. Resuspend KREA*pure* column material using a vortex.
2. Loosen cap ¼ turn and snap off the bottom closure.
3. Place the column in a 2 mL collection tube (not provided).
4. Pre-centrifuge the column for 1 min at 20,800 x g (i.e. max speed of a typically tabletop microcentrifuge).
5. Discard flow-through and column cap, but re-use collection tube.
6. Add 300 µL ultrapure water to the column and centrifuge for 1 min max speed using a tabletop centrifuge.
7. Discard collection tube and flow-through.

8. Place column into a new RNAse-free 1.5 mL microcentrifuge tube (not provided).
9. Add ULS-labeled sample onto the center of the column bed.
10. Centrifuge column for 1 min at max speed using a tabletop microcentrifuge.
11. Flow-through contains the purified labeled total RNA.

IV. Determination of Degree of Labeling (DoL)

12. Determine the degree of labeling (DoL) as follows: measure absorbance at 260 nm and 550 nm (for Cy3-ULS) or 650 nm (for Cy5-ULS) using a spectrophotometer (e.g. Nanodrop).
13. Calculate the DoL value as explained in the box or use the interactive calculator on our web site (www.kreatech.com).

Degree of Labeling (DoL) calculation

$$x = \text{ng (nucleic acid)} / \mu l = \frac{(OD_{260} - (OD_{dye} * \text{corr. factor})) * \text{dilution factor} * 40}{\text{cuvette path length (cm)}}$$

$$y = \text{pmol (dye)} / \mu l = \frac{OD_{dye} * \text{dilution factor}}{\text{cuvette path length (cm)} * \varepsilon dye * 1 \times 10^{-6}}$$

$$DOL = \frac{340 * y * 100\%}{x * 1000}$$

εdye Cy3-ULS = 150,000 $mol^{-1} * L * cm^{-1}$
εdye Cy5-ULS = 250,000 $mol^{-1} * L * cm^{-1}$
Correction factor for Cy3 = 0.08
Correction factor for Cy5 = 0.05

NOTE: *The recommended DoL value is 2-3% (indicating an average of 2-3 Cy-ULS molecules per 100 nucleotides). It is advised to use the material for microarray hybridization only when the DoL value is between 1.0-3.6%. DoL values*

lower than 1.0% may not produce enough signal, whereas DoL values higher than 3.6% might cause either high background levels or quenching of signal. In these cases please refer to the trouble shooting section or contact us at techservices@kreatech.com.

V. Preparation of labeled total RNA using KREA*block* (optional) solution for hybridization

1. Pool the labeled samples.
2. Concentrate (using a concentrator) to nearly dryness.
   NOTE: *It is important not to overdry the labeled material.*
3. After concentration, dissolve the labeled material in ¼ volume RNAse-free water and add ¼ volume KREA*block* (optional; otherwise add another ¼ volume ultrapure water).
   NOTE: *Be sure that no precipitates of labeled material will be present in the samples (this can cause background on the slides). If so, warm the samples at 42 ºC and dissolve again.*
4. Add ½ volume of 2 x Hybridization buffer.

Important remarks:

1. KREA*block* is a 4 x soluition and should be added as ¼th of the final volume of the hybridization mixture (e.g. 25 µL of KREA*block* in a 100 µL hybridization volume).
2. Hybridize and wash slides according to own protocol (we recommend using the provided KREA*block* solution to moisturize the hybridization chamber).

D. Protocol for using microRNA enriched RNA

Before you begin

See below procedural notes that you should read and understand before starting an experiment.

I. Procedural Notes

Components to perform the enrichment procedure are not provided in this kit. If enrichment is required we recommend using a method designed for purifying and enriching small RNA from samples of tissue or cultured cells that will result in minimal contamination from large RNA molecules or genomic DNA.

Enrichment methods provided that have been tested in combination with the ULS technology are: miRNeasy™ procedure (QIAGEN), *mir*Vana™miRNA Isolation procedure (Ambion/Applied Biosystems), miRACLE™ miRNA isolation kit (Stratagene).

II. ULS Labeling

1. Determine the concentration of the small RNA by absorbance measurement at 260 nm. Also measure at 230 and 280 nm to determine the purity of the small RNA sample (see Procedural Notes for the quality criteria).
2. Integrity of the small RNA might be assessed by denaturing polyacrylamide gel electrophoresis.
3. When small RNA meets the quality criteria, set up labeling reactions in RNAse-free PCR tubes as described below (use 1.5 mL microcentrifuge tubes when using a water bath).

Degree of Labeling (DoL) calculation

$$x = \text{ng (nucleic acid)} / \mu l = \frac{(OD_{260} - (OD_{dye} * \text{corr. factor})) * \text{dilution factor} * 40}{\text{cuvette path length (cm)}}$$

$$y = \text{pmol (dye)} / \mu l = \frac{OD_{dye} * \text{dilution factor}}{\text{cuvette path length (cm)} * \varepsilon dye * 1 \times 10^{-6}}$$

$$D0L = \frac{340 * y * 100\%}{x * 1000}$$

εdye Cy3-ULS = 150,000 $mol^{-1} * L * cm^{-1}$
εdye Cy5-ULS = 250,000 $mol^{-1} * L * cm^{-1}$
Correction factor for Cy3 = 0.08
Correction factor for Cy5 = 0.05

NOTE: *The procedure described below is assuming the need to hybridize 1 µg of miRNA enriched RNA per sample on a microarray. In case your application needs less or more sample to be hybridized, you can scale down or up accordingly: use a ratio of exactly 1.0 µl of ULS per 1.0 µg of enriched RNA. The optimal RNA concentration for ULS labeling is ≥ 50 ng/µL. If no 1 µg RNA is available use lower labeling volumes in order to aim for a RNA concentration of 50 ng/µL (e.g. if only 250 ng RNA is available the optimal labeling volume is 5 µL). The reagents included in the kit allow you to use up to 2.5 µg of miRNA enriched RNA per labeling reaction.*

a. Pre-heat a waterbath OR thermocycler at 85°C.
b. Mark RNAse-free PCR tubes or 1.5 mL microcentrifuge tubes to identify samples. Use one tube per samples..
c. Add 1 µg of RNA and ultrapure water to the tube to a total volume of 17.0 µl
d. Add 2.0 µl of 10 x Labeling Solution

e. Add 1.0 µl of Cy3-ULS or Cy5-ULS
f. Mix by pipetting

Example of labeling 1 µg microRNA enriched RNA.

	Sample 1	Sample 2
miRNA enriched RNA (1 µg) + ultrapure water	17 µL	17 µL
Cy3-ULS	1.0 µL	-
Cy5-ULS	-	1.0 µL
10 x Labeling Solution	2.0 µL	2.0 µL
Total volume	20 µL	20 µL

4. Incubate for 15 minutes at 85°C in a PCR machine with hot lid (or a pre-warmed water bath).
5. During the final 10 minutes of this incubation, preparation of the KREA*pure* column can be done (see section III below).
6. Put the sample on ice for at least 1 minute.

   OPTIONAL: *If lower labeling volumes are used adjust volume to 20 µL using ultrapure water.*

III. Removal of non-reacted Cy-ULS using KREA*pure* columns

1. Resuspend KREA*pure* column material using a vortex.
2. Loosen cap ¼ turn and snap off the bottom closure.
3. Place the column in a 2 mL collection tube (not provided).
4. Pre-centrifuge the column for 1 min at 28,000 x g (i.e. max speed of a typical tabletop microcentrifuge
5. Discard flow-through and column cap, but re-use collection tube.

6. Add 300 µL ultrapure water to the column and centrifuge for 1 min at max speed using a tabletop centrifuge.
7. Discard collection tube and flow-through.
8. Place column into a new RNAse-free 1.5 mL micro-centrifuge tube (not provided).
9. Add ULS-labeled total RNA onto column bed.
10. Centrifuge column for 1 min at max speed using a tabletop centrifuge.
11. Flow-through contains the purified labeled miRNA.

IV. Determination of Degree of Labeling

12. Determine the degree of labeling (DoL) as follows: measure absorbance at 260 nm and 550nm (for Cy3-ULS) or 650 nm (for Cy5-ULS) using spectrophotometer (e.g. Nanodrop).
13. Calculate the DoL value as explained in the box below or use the interactive calculator on our web site (www.kreatech.com).

NOTE: *The recommended DoL value is 2-3% (indicating an average of 2-3 Cy-ULS molecules per 100 nucleotides). It is advised to use the material for microarray hybridization only when the DoL value is between 1.0-3.6%. DoL values lower than 1.0% may not produce enough signal, whereas DoL values higher than 3.6% might cause either high background levels or quenching of signal. In these cases please refer to the trouble shooting section or contact us at techservices@kreatech.com.*

V. Preparation of labeled microRNA enriched RNA using KREA*block* (optional) solution for hybridization

1. Pool the labeled samples.
2. Concentrate (using a concentrator) to nearly dryness.
   NOTE: *It is important not to overdry the labeled material.*

3. After concentration, dissolve the labeled material in ¼ volume RNAse-free water and add ¼ volume KREA*block* (optional; otherwise add another ¼ volume RNAse-free water).
   NOTE: *Be sure that no precipitates of labeled material will be present in the samples (this can cause background on the slides). If so, warm the samples at 42 ºC and dissolve again.*
4. Add ½ volume of 2 x Hybridization buffer.

Important remarks:

1. KREA*block* is a 4 x solution and should be added as ¼th of the final volume of the hybridization mixture (e.g. 25 µL of KREA*block* in a 100 µL hybridization volume).
2. Hybridize and wash slides according to own protocol (we recommend that the KREA*block* solution be used to provide the moisture in the hybridization chamber).

E. Trouble Shooting

I. ULS Labeling

Observation	Possible Cause	Suggestions
Degree of labeling is too low	Salt present which disturbs labeling	Repeat purification procedure or precipitate the RNA and dissolve in ultrapure water
	Incorrect ratio of labeling reagent to RNA	Use exactly 1.0 µl of ULS per 1.0 µg of RNA
	RNA concentration during labeling reaction too low	Ensure concentration of the labeling is above 50ng/µl
Degree of labeling is too high	Incorrect ratio of labeling reagent to RNAs	Ensure use of instructed amount of ULS per µg of RNAs

II. Array hybridization and detection

Observation	Possible Cause	Suggestions
Background on the slide	Too much sample added to the microarray	Reduce sample amount
	Insufficient blocking	Add more or alternative blockers and/or KREA*block* to pre-hybridization or hybridization buffer
	Partial drying of hybridization buffer during hybridization due to low humidity in the hybridization chamber	Ensure sufficient moisture is added to hybridization chamber and vessel is sealed tightly. KREA*block* can be used to moisturize the hybridization chamber

F. Appendix

I. Determination of RNA Quality

- Measure absorbance at 230 nm, 260 nm and 280 nm using a spectrophotometer.
- Calculate A_{260}/A_{280} and A_{260}/A_{230} ratios
- Good quality RNA the $A_{260}/_{280}$ should be between 1.9 and 2.1.
- The $A_{260/230}$ should be above 2.1.

II. Determination of the RNA Concentration

- Measure absorbance at 260 nm
- Calculate RNA concencentration:

$$\text{ng nucleic acid / µl} = \frac{A_{260} * \text{dilution factor} * 40}{\text{cuvette length (in cm)}}$$

III. RNA Qualification

The protocol provided is to analyze the small RNA preparation on a 15% polyacrylamide TBE-urea gel.

1. Assemble a 15% polyacrylamide TBE-urea gel with 1× TBE running buffer (see below).
2. Combine 5 µL 2 × TBE-urea sample loading buffer (see below) with 25-100 ng or up to 5 µL of the small RNA preparation. If needed, adjust the volume to 10 µL with H_2O.
3. Combine 5 µL 2 × TBE-urea sample loading buffer and 50 ng of a 10-bp DNA ladder. Adjust the volume to 10 µL with H_2O.
4. Heat the samples at 70°C for 3 minutes.
5. Flush wells of the gel repeatedly to remove all stray acrylamide or urea from the wells and then load the samples on the gel.
6. Perform electrophoresis at 180V for 60–75 minutes or until the bromophenol blue dye reaches the bottom of the gel.
7. Stain the gel using SYBR® Gold or ethidium bromide and visualize the stained nucleic acids using a UV-transillumintor.

<table>
<tr><th>15% Polyacrylamide TBE-Urea Gel (per 15 mL Gel Solution)</th><th>2 × TBE-Urea Sample Loading Buffer (per 10 mL)</th></tr>
<tr><td rowspan="3">1.5 mL of 10 × TBE buffer
5.6 mL of 40% acrylamide solution (29:1 acrylamide:bis-acrylamide)
7.2 g urea
1.9 mL H_2O
Heat to 37°C while stirring (until urea is dissolved)
Cool to room temperature
Immediately before pouring the gel, add the following reagents:
7.5 µL of TEMED
75 µL of 10% ammonium persulfate</td><td>1 mL of 10 × TBE Buffer
0.01% bromophenol blue
0.05% xylene cyanol
1.2 g Ficoll
4.2 g urea
Add deionized H_2O to a final volume of 10 mL</td></tr>
<tr><th>10 × TBE Buffer (per Liter)</th></tr>
<tr><td>108.0 g of Tris base
5.8 g of EDTA (free acid)
55.0 g of boric acid
Add deionized H_2O to a final volume of 1 liter</td></tr>
</table>