VMRD

Nucleic Acid Extraction Kit

User manual

Magnetic Bead

Table of contents

1. **Product description**

This Nucleic Acid Extraction Magnetic Bead Kit is designed for Manual **or** Automated extraction of nucleic acids in bodily fluids such as blood, serum, plasma, semen, milk, and saliva, as well as swabs, feces and tissue samples. For sample compatibility see Section 4.

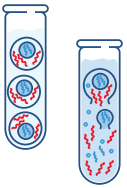
The principles behind Magnetic Bead Extraction are Lyse, Bind/Adsorb, Separate, Wash, Elute.

- Nucleic acids are released from the sample upon lysis with the lysis master mix (SLS or ELS) containing Proteinase K.
- Binding Buffer and the Beads are added to the lysate to facilitate nucleic acid binding to the beads.
- Nucleic acids become adsorbed/bound to the paramagnetic beads. The beads are then separated from the remaining sample material by applying magnetic field to the tubes or plate. (In automated plate systems, the beads are collected with 96 magnetized rods/pins.)
- After magnetic separation, unbound contaminants and salts are washed away with Wash Buffers 1 and 2, then 80% ethanol. Residual ethanol is removed by brief air drying of the separated beads.
- Purified nucleic acids are eluted from the magnetic beads under low salt conditions.
- Nucleic acids are ready-for-use in downstream reactions.

1.1. Overview of the Procedure

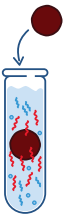
1. Lyse Sample

Sample lysis occurs in the presence of chaotropic ions and Proteinase K



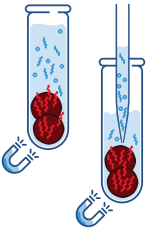
2. Bind Nucleic Acids

Add Binding Working Mix to Lysate. Mix/Shake then **Incubate** to allow adsorption of nucleic acids to paramagnetic beads.



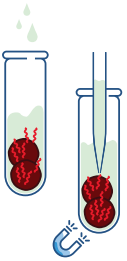
3. Separate Beads

Apply magnetic field to collect/isolate magnetic beads for each subsequent step.



4. Wash & Dry

Perform clean-up of nucleic acids bound to paramagnetic beads with Wash buffer 1, Wash buffer 2 and 80% Ethanol



5. Elute Nucleic Acids

Add 50-100 µL low-salt elution buffer. **Incubate**. Separate beads a final time. Collect elution liquid.

1.2. Specifications of the Kit

This Magnetic Bead Extraction Kit is designed for manual or automated preparation of viral RNA/DNA from serum or plasma, blood, homogenized tissue, ear notches, stool/feces, swabs, milk, and oral fluids. The kit is designed for use in manual mode in 96-well plates with a magnetic separator (see VMRD product MOL-EXTRACT-SEPARATOR) or with automated magnetic separation systems using pins or rods.

- Time for manual completion of 96 samples is around 120 minutes.
- Time for automated completion of 96 samples depends upon chosen platform/instrument.
- The prepared nucleic acids are suitable for applications, such as automated fluorescent DNA sequencing, RT-PCR, PCR, or any kind of enzymatic reaction.
- Well-to-well consistency relies upon complete resuspension of beads prior to use.
- Premixing magnetic beads with the binding buffer allows easier homogenous distribution of the beads to the individual wells of the separation plate. During automation, a premix step before aspirating the beads/binding buffer mixture from the reservoir is recommended to keep the beads resuspended.
- Resuspension by pipetting up and down is more efficient than mixing by a shaker or magnetic mixer. Simultaneous mixing of all samples on a shaker may save time, but the possible negative effect of suboptimal mixing needs to be considered and experimentally determined.

Kit specifications at a glance

Parameter	
Technology	Magnetic Bead technology
Format	96-well plate
Sample material	up to 200 µL serum, plasma, blood, bacterial culture, homogenized tissue, ear notches, stool/feces, swabs, milk, oral fluids/saliva
Fragment size	approx. 300 bp-50 kb
Elution volume	50-100 µL
Preparation time	120 min/96 preps (manual) or about 45-60 min/96 preps (automated)

2. Components

Store all components at room temperature (18-25°C)

2.1. Kit components and Storage Conditions

- All kit components can be stored at room temperature (18-25°C) and are stable for up to 15 months from the date of manufacturing.
- After reconstitution of Carrier RNA, the solution should be stored at <-10°C in small aliquots.
- All buffers are delivered ready-to-use.

Component	Quantity
SLS (Standard Lysis Solution)	30 mL
ELS (Enhanced Lysis Solution)	60 mL
Carrier RNA	2 x 400 µg
Carrier RNA Diluent	2 x 0.5 mL
Magnetic Beads	3 mL
Binding Buffer	110 mL
Proteinase K	5 mL
Wash Buffer 1	75 mL
Wash Buffer 2	75 mL
Elution Buffer	30 mL
User Manual	1

2.2. Materials supplied by the User

All materials must be nuclease-free!

Reagents

- 1X PBS pH 7.2-7.4
- Saline
- Culture medium
- Nuclease-free H_2O
- DNA or RNA extraction controls (EC)
- 80% nuclease-free ethanol
- Lysis Beads (cat: MOL-EXTRACT-BEADS)

Consumables

- Filter tips
- Tubes and microtubes
- Deep-well 96-well plates
- 96-well micro plates
- 96-tip combs
- Reservoirs
- Other equipment specific consumables

Equipment

- Benchtop centrifuge
- Bead beater or plate shaker
- Vortex or another lab mixer
- Tissue grinder
- Pipettes
- Magnetic separator (see below)
- Heat block or water bath

Magnetic separation systems

This Nucleic Acid Extraction Kit is compatible with manual and automated magnetic bead separation systems. It is recommended that for manual extraction a magnetic separator that ensures complete separation of the beads is chosen. The recommended manual separator is available through the VMRD website (cat: MOL-EXTRACT-SEPARATOR).

3. Need to know before starting

Precautions

- **Proteinase K is sensitive to chaotropic ions** in the lysis solutions (SLS and ELS) and must be added to the lysis working mix **immediately prior to use**. Do not expose proteinase K to the lysis solution for extended period of time.
- **SLS and ELS cannot be used interchangeably**. SLS should provide sufficient lysis for most sample types. It is recommended that ELS is used for samples suspected to contain high concentration of inhibitors.
- Dissolve 1 vial of Carrier RNA (400 µg) in 500 µL of Carrier RNA Diluent. It is recommended that reconstituted Carrier RNA be stored at <-10˚C in small aliquots to prevent degradation due to multiple freeze-thaw cycles.
- If using automated magnetic bead handling equipment, download the appropriate program from vmrd.com and install.

Determine shaker settings

Determine appropriate shaker speed for the **binding step of the manual procedure**:

1. Load 1.1 mL water (total volume of binding step) to the wells of a deep-well plate. A dye of choice may be added to the water for easier visualization. Cover the plate with tape and place on the shaker. Start shaking with a moderate speed setting for 15 seconds. Turn off the shaker and check the tape surface for small droplets of dyed water.
2. Increase speed setting, shake for additional 15 seconds, and check the tape surface for droplets again.
3. Continue increasing the speed setting until you observe droplets on the surface of the tape. Reduce speed setting, check again, and use this setting for the wash step.

To adjust shaker setting for **wash and elution**, repeat the above steps with **600 µL and 100 µL** of dyed water, respectively.

Determine optimal magnetic bead separation time

Complete attraction of the magnetic beads to the pins of a magnetic separator depends on the strength of the magnet, thickness of the selected separation plate, distance of the separation plate from the magnetic pins, and the volume to be processed. The time needed for attraction of the beads to the magnetic pins should be adjusted by the end user to assure the complete attraction of the beads. Contact customer support at support@vmrd.com for recommendations for separation equipment.

4. Protocols

4.1. How to navigate this Manual

1. **Identify** an appropriate **protocol** according to section 4.2.
2. **Prepare samples** according to section 4.3.
3. Prepare Sample **Lysis Working Mix** and **Magnetic Beads Binding Working Mix** according to the appropriate table in section 4.4.
4. **Lyse samples** according to notes in appropriate table in section 4.4.
5. **Adsorb released nucleic acids** to the paramagnetic beads according to procedure in section 4.5 (manual procedure) or section 4.8 (for automated procedure using KingFisher® Flex). For all other systems contact VMRD technical support at support@vmrd.com.
6. **Wash out contaminants and elute nucleic acid** according to protocol in section 4.6-7 (for manual procedure) or section 4.8-9 (for automated procedure using KingFisher® Flex). For all other systems contact VMRD technical support at support@vmrd.com.

4.2. Identification of Appropriate Protocol

step 1 for all protocols

This Nucleic Acid Extraction Kit is suitable for purification of DNA and RNA from various sample types. It is vital that the correct protocol is identified prior to starting the extraction procedure.

Use the table below or contact support@vmrd.com to identify optimal protocol. The protocol may require modification and validation to fit individual needs.

S

Standard

for Viral Nucleic Acids, Bacterial Nucleic Acids

- Blood
- Plasma
- Serum
- Milk
- Oral fluids
- Ear notch in PBS
- Tissue homogenized in PBS

E

Enhanced

for Nucleic Acids from Difficult to Lyse Bacteria including mycobacterium

- Blood
- Plasma
- Serum
- Milk
- Oral fluids
- Ear notch in PBS
- Tissue homogenized in PBS

M

Modified

for Viral Nucleic Acids

- Ear notch in SLS
- Tissue homogenized in SLS
- Ear notch in ELS
- Tissue homogenized in ELS

HB

High Inhibitors

for Nucleic Acids from Difficult to Lyse Bacteria

- Feces

HV

High Inhibitors Viral Nucleic Acids

for Viral Nucleic Acids

- Feces

HE

High Extenders

for Viral and Bacterial Nucleic Acids

- Semen

Remember correct symbol color!

4.3. Preparation of Samples

step 2 for all protocols

Sample types	Sample preparation procedure	Protocol
Blood, serum, plasma, urine, oral fluids, milk	Up to 200 µL of a neat OR diluted sample can be used. **Do not use larger volumes**. If poor recovery of DNA from milk is observed, addition of 1% Tween-20 to PBS used in the purification procedure (enhanced protocol) may improve the yield.	S E
Semen samples	If intracellular nucleic acids are being purified, it is recommended that the liquid portion of the semen is removed by centrifugation at 15,000 x g for 2 min and only pelleted cells are used in the procedure. If target nucleic acids are expected to be extracellular, 200 µL of the whole semen (neat or diluted in a neutral buffer such as PBS) can be used for the lysis.	HE
Fecal samples	Mix 0.3 g of feces with 1 mL of PBS, then vortex and proceed as on page 14. Sample mass and volume of PBS may be increased proportionally, depending on the internal standard operating procedures.	HB HV
Difficult to lyse bacteria	Purification of nucleic acids from difficult to lyse bacteria from any sample matrix requires physical disruption using glass beads. To lyse the bacteria, 200 µL of undiluted sample or sample diluted in a neutral buffer such as PBS should be processed according to the enhanced protocol.	E

Sample types	Sample preparation procedure	Protocol
Tissue samples	Homogenize tissue samples according to internally accepted standard operating procedure. Typically, 5–25 mg sample material can be homogenized in at least 200 µL of PBS, SLS or ELS buffer using a bead-based homogenizer, tissue grinder or other tissue disruption method. It should be considered that the copurified total nucleic acid may cause inhibition in subsequent PCR assays. After homogenization of the tissue, allow the tissue to settle or centrifuge at low speed to clarify the lysate. Use up to 200 µL of clear supernatant for the appropriate purification protocol. If using less than 200 µL of sample, adjust with PBS buffer to a final volume of 200 µL.	S E M
Skin punches and notches	If skin punches are used for testing, skin sections that are minimum 2-3 mm in diameter or up to 1 cm long should be submerged in at least 200 µL of PBS, SLS or ELS buffer according to the Modified protocol and incubated at room temperature for a minimum of 15 min. Extending the incubation time up to 24 h is acceptable.	S E M
Swab samples	Dip the swab in a neutral buffer such as PBS, saline or cell culture medium and vortex briefly. Alternatively allow the swabs to be submerged in neutral buffer or culture media for minimum 15 min with shaking. Remove and squeeze out the swab. Proceed to the extraction protocol with 200 µL of the particle-free buffer.	E HB HV

4.4. Preparation of Lysis & Binding Working Mixes

Important notes

- **Proteinase K is sensitive to chaotropic salts** and should not be exposed to the SLS or ELS for extended time.
- Prepare Lysis Working Mix according to the appropriate table in this section **immediately before use**.
- Homogeneous distribution of magnetic beads is essential for a sample-to-sample consistency. Therefore, the magnetic bead suspension must be vigorously shaken immediately prior to use.
- Prepare Binding Working Mix according to the appropriate tables on the following pages. Mix the binding working mix by vortexing immediately prior to adding to sample wells/tubes. During automation, a premix step before aspirating the beads/binding buffer mixture from the reservoir is recommended to keep the beads re-suspended.
- It is recommended that 10% overage is calculated into the final volume, to account for transfer loss.

Lysis Working Mix S

step 3 for standard

Reagent	**Volume** required per sample
Standard Lysis Solution	180 µL
Proteinase K	20 µL
Carrier RNA (recommended)	4 µL
Extraction Control (recommended)	x µL
Total volume	200 µL + recommended reagents

Binding Working Mix S

Reagent	**Volume** required per sample
Binding Buffer	600 µL
Magnetic Beads	20 µL
Total volume	620 µL

Lysis procedure S

step 4 for standard

1. Combine 200 µL of sample with 200 µL (+ volume of recommended reagents) of Lysis Working Mix.
2. Mix thoroughly by vortexing or pipetting.
3. Use the entire volume of the lysate in the Magnetic Bead binding reaction in step 4.5 or 4.8.

Lysis Working Mix E

step 3 for enhanced

Reagent	**Volume** required per sample
Enhanced Lysis Solution	350 µL
1X PBS	150 µL
Carrier RNA (recommended)	7 µL
Extraction Control (recommended)	x µL
Total volume	500 µL + recommended reagents

Binding Working Mix E

Reagent	**Volume** required per sample
Binding Buffer	600 µL
Proteinase K	20 µL
Magnetic Beads	20 µL
Total volume	640 µL

Lysis procedure E

step 4 for enhanced

1. Combine 200 µL of sample with 500 µL (+ recommended reagents) of Lysis Working Mix in a vial with Lysis Beads.
2. Shake the sample vigorously for 15 min using a bead beater, vortexer or equivalent equipment.
3. Clarify the lysate by centrifugation at 16,000 x g for 5 min.
4. Use 405 µL of the clarified lysate in the Magnetic Bead binding reaction in step 4.5 or 4.8.

Lysis Working Mix M

step 3 for modified

Reagent	**Volume** required per sample
SLS or **ELS**	200 µL
Carrier RNA (recommended)	4 µL
Total volume	200 µL + recommended reagents

Binding Working Mix M

Reagent	**Volume** required per sample
Binding Buffer	600 µL
Proteinase K	20 µL
Magnetic Beads	20 µL
Extraction Control (recommended)	x µL
Total volume	640 µL + recommended reagents

Lysis procedure M

step 4 for modified

1. Completely submerge an appropriately sized tissue section in 200 µL (+ recommended reagents) of Lysis Working Mix.
2. Incubate the sample at room temperature for a minimum of 15 min. Extending the incubation time up to 24 h is acceptable.
3. Some tissues may be mechanically homogenized with SLS or ELS
4. Use the entire liquid portion of the lysate in the Magnetic Bead binding reaction in step 4.5.

Lysis Working Mix HB

step 3
for high inhibitors

Reagent	**Volume** required per sample
Enhanced Lysis Solution	350 µL
1X PBS	210 µL
Carrier RNA (recommended)	7 µL
Extraction Control (recommended)	x µL
Total volume	560 µL + recommended reagents

Binding Working Mix HB

Reagent	**Volume** required per sample
Binding Buffer	600 µL
Proteinase K	20 µL
Magnetic Beads	20 µL
Total volume	640 µL

Lysis procedure HB

step 4
for high inhibitors

1. Re-suspend minimum 0.3 g feces in PBS at ratio 1 mL PBS per 0.3 g feces.
2. Mix samples vigorously (vortex) for 3 min. Pellet the debris by centrifugation at 100 x g for 5 min.
3. Combine 140 µL of clarified sample supernatant with 560 µL (+ recommended reagents) of Lysis Working Mix in a vial with Lysis Beads.
4. Shake the sample vigorously for 15 min using a bead beater, vortexer or equivalent equipment.
5. Clarify the lysate by centrifugation at 16,000 x g for 5 min.
6. Use 405 µL of the clarified lysate in the Magnetic Bead binding reaction.

Lysis Working Mix HV

step 3
for high inhibitors viral nucleic acids

Reagent	**Volume** required per sample
Enhanced Lysis Solution	350 µL
Carrier RNA (recommended)	7 µL
Extraction Control (recommended)	x µL
Total volume	350 µL + recommended reagents

Binding Working Mix HV

Reagent	**Volume** required per sample
Binding Buffer	600 µL
Proteinase K	20 µL
Magnetic Beads	20 µL
Total volume	640 µL

Lysis procedure HV

step 4
for high inhibitors viral nucleic acids

1. Re-suspend minimum 0.3 g feces in PBS at ratio 1 mL PBS per 0.3 g feces.
2. Mix samples vigorously (vortex) for 3 min. Pellet the debris by centrifugation at 100 x g for 5 min.
3. Combine 140 µL of clarified sample supernatant with 350 µL (+ recommended reagents) of Lysis Working Mix in a vial.
4. Mix vigorously by pipetting or vortexing.
5. Clarify the lysate by centrifugation at 16,000 x g for 5 min.
6. Use 405 µL of the clarified lysate in the Magnetic Bead binding reaction.

Lysis Working Mix HE

step 3 for high extenders

Reagent	**Volume** required per sample
Enhanced Lysis Solution	200 µL
Proteinase K	20 µL
Carrier RNA (recommended)	4 µL
Total volume	220 µL + recommended reagents

Binding Working Mix HE

Reagent	**Volume** required per sample
Binding Buffer	600 µL
Magnetic Beads	20 µL
Extraction Control (recommended)	x µL
Total volume	620 µL + recommended reagents

Lysis procedure HE

step 4 for high extenders

1. Combine 200 µL of semen prepared in section 4.3 with the Lysis Working Mix. Vortex or thoroughly mix by pipetting and incubate at room temperature for 15 min.

   Optional: Increasing the incubation temperature to 60°C should improve the yield and purity of purified nucleic acids.

2. Clarify the lysate by centrifugation at 15,000 x g for 1 min and use the entire clarified supernatant in the Magnetic Bead binding reaction in step 4.5.

4.5. Binding of Nucleic Acids to Beads

step 5 of manual procedure

1. Add the appropriate volume (as specified in corresponding tables of section 4.4) of **Binding Working Mix** into the processing tubes/deep wells.
2. **Transfer the lysates** (as specified in section 4.4) into the tubes/deep wells with Binding Working Mix.
3. **Mix thoroughly** by repeated pipetting and shake for 5-10 min at room temperature. Alternatively, when processing the samples without a shaker, pipette up and down, at least 10 times and incubate for 5-10 min at room temperature.
4. **Separate the magnetic beads** by placing the deep-well plate/tubes on a magnetic separator. Wait at least 2 min until all beads have been completely attracted to the magnet. Without removing the plate from the magnetic separator, aspirate and discard the supernatant.

   Do not disturb the attracted beads.

4.6. Washing and drying of Nucleic Acids Bound to Beads

step 6 of manual procedure

1. **Washing with Wash Buffer 1**

   1.1. Remove the processing tubes/deep-well plate from the magnetic separator.
   1.2. Add 600 µL Wash Buffer 1 and re-suspend the beads by shaking or pipetting until the beads are re-suspended completely (1–3 min).
   1.3. Separate the magnetic beads as in step 4.5.4.

2. **Washing with Wash Buffer 2**

   2.1. Remove the deep-well plate/tubes from the magnetic separator.
   2.2. Add 600 µL Wash Buffer 2 and re-suspend the beads as in step 4.5.3.
   2.3. Separate the magnetic beads as in step 4.5.4.

3. **Washing with 80% Ethanol**

   3.1. Remove the deep-well plate from the magnetic separator.
   3.2. Add 600 µL 80% ethanol and re-suspend the beads as in step 4.5.3.
   3.3. Separate the magnetic beads as in step 4.5.4.

4. **Air-dry Magnetic Beads**

   Air-dry the magnetic bead pellet for at least 10 min at room temperature. This time can be increased up to 30 min. Complete removal of ethanol is critical for downstream applications.

4.7. Elution of Nucleic Acids

step 7 of manual procedure

1. **Remove** the processing tubes/deep-well plate from the magnetic separator.
2. Add 50-100 µL **Elution Buffer** to the processing tubes/deep-well plate and re-suspend the beads by pipetting or shaking for 1-3 min at room temperature.
   - Incubation of beads with the Elution Buffer for 5 min at 56˚C may increase the yield of purified nucleic acids.
   - It is essential to cover beads completely with Elution Buffer during the elution step.
3. **Separate magnetic beads** by placing the processing tubes/plates on a magnetic separator as in step 4.5.4.
4. **Collect and Transfer** supernatant containing nucleic acids to plates or tubes for storage or downstream analysis.

Purified nucleic acids can be used for further downstream reactions.

Please continue to page 19 for automated procedure (sections 4.8 and 4.9).

4.8. Binding and Elution of Nucleic Acids

step 5 of automated procedure

1. **Set up the KingFisher® Flex** instrument according to section 4.9.
2. **Transfer the lysates** (as specified in section 4.4.) into the wells of a deep well plate.
3. Add the appropriate volume of **Binding Working Mix** (as specified in the corresponding tables of section 4.4.) into the wells with lysates.
4. Transfer the plate into the KingFisher® Flex instrument.
5. **Start** the Nucleic Acid Extraction program.
6. The instrument stops after the final elution step. Follow the instructions on the instrument´s display and unload the plates.

   Purified nucleic acids can be used for further PCR analysis.

4.9. Preparation of KingFisher® Flex

prep for step 5 of automated procedure

1. Start the KingFisher® Flex instrument and choose the applicable protocol. Follow the instructions on the screen to prepare the plates.
2. Add 50-100 µL **Elution Buffer** into the wells of an empty 96-well plate.
3. Add 600 µL **80% ethanol** into the wells of an empty 96-well deep-well plate.
4. Add 600 µL **Wash Buffer 2** into the wells of an empty 96-well deep-well plate.
5. Add 600 µL **Wash Buffer 1** into the wells of an empty 96-well deep-well plate.

5. Safety instructions

- Always wear appropriate personal protection equipment including, but not limited to: lab coat, disposable gloves, and protective goggles.
- **DO NOT add bleach** or acidic solutions to waste containing guanidine hydrochloride, as the reaction may result in the production of highly reactive compounds.
- The following components of the kit contain hazardous materials:
  - Standard Lysis Solution (SLS): Guanidine hydrochloride, 36–<50%; CAS 9003-98-9
  - Enhanced Lysis Solution (ELS): Guanidinium thiocyanate, 45-<60%; CAS 593-84-0
  - Carrier RNA Diluent: Guanidinium thiocyanate, 30-<45%; CAS 593-84-0
  - Binding Buffer: Sodium perchlorate, 15–40%; CAS 7601-89-0, Ethanol, 35-<55%; 64-17-5
  - Wash Buffer 1, Wash Buffer 2: Sodium perchlorate, 15-<40% CAS 7601-89-0; Ethanol 20–<35%; CAS 64-17-5

For more information, please refer to the appropriate safety data sheets (SDSs).

6. Troubleshooting

Low quantity of purified nucleic acids

- Use the alternative lysis solution (SLS or ELS), include 1% Tween in PBS or incubate the sample in the lysis solution at 60˚C if insufficient sample lysis is suspected.
- For particularly difficult samples TRIzol® lysis may be required. Add 1 mL of TRIzol® to 10–30 mg tissue or up to 250 µL blood and homogenize. Use the aqueous, colorless (upper) phase in the appropriate protocol.
- Bead pellet must be covered completely with elution buffer and shaken vigorously to ensure complete dissociation of purified nucleic acids from the beads.
- Increasing the temperature of the elution step to 56˚C may improve yield.
- Ensure complete removal of wash buffers after each step.
- Prevent magnetic beads from drying out.
- Ensure complete separation of magnetic beads and prevent removal of unattracted beads during aspiration by choosing a stronger magnet or increasing incubation time.
- Do not disturb magnetic bead pellet during aspiration steps.
- Decrease aspiration speed to prevent bead removal.

Low purity of nucleic acids

- Shake the magnetic beads vigorously to ensure complete resuspension of the beads and sufficient washing.
- Ensure complete removal of wash buffers after each step.
- Always use the appropriate combinations of separators and plates.

Poor performance in downstream applications

- Allow the 80% ethanol to evaporate completely as leftover ethanol may interfere with downstream applications.
- Never reuse buffers from buffer reservoirs.
- Store all components of the kit in tightly closed bottles to prevent ethanol evaporation.

Molecular
vmrd.com

Version 3

VMRD, Inc. Pullman, WA 99163, USA