

Research Adjuvants

Step-by-Step Instructions

TiterMax® Research Adjuvants combine the benefits of potent synthetic copolymers, CRL-8941 in **TiterMax® Classic** and CRL-8300 in **TiterMax® Gold**, with those of a microparticulate-stabilized, water-in-oil emulsion containing a metabolizable non-toxic oil, squalene. Emulsions of **TiterMax®** can be prepared using any technique which works with Freund's Complete Adjuvant (FCA). This pamphlet describes several methods from which you may choose, depending on the equipment you have available and the volume of emulsion you are going to prepare. The two methods we find to be the best in terms of simplicity and recovery of emulsion are the two syringe, double hub needle method and the Kontes Pellet Pestle® homogenizer method. The latter method is especially suited to small volumes. A number of other methods suggested by recent **TiterMax®** users are also described. Each of these methods will produce stable water-in-oil emulsions in 1 to 5 minutes for **TiterMax® Classic** and **TiterMax® Gold** adjuvants. Please keep in mind that **TiterMax® Gold** requires larger volumes for immunization.

Stable water-in-oil emulsions are notoriously difficult to make. The **TiterMax®** formulation was developed to produce a very stable emulsion. For a detailed discussion of emulsion preparation theory, please refer to our Technical Brochure. The **TiterMax®** line of adjuvants were developed to meet the specific needs of investigators for an immunoadjuvant that is at least as effective as Freund's Complete Adjuvant (FCA), and safer and easier to use. Like FCA, **TiterMax®** adjuvants can be used with a wide variety of antigens. Please follow the step-by-step instructions carefully for the method you choose.

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Method 1: Two-Syringe, Double Hub Needle

This method is suitable for emulsion volumes between 1 ml and 10 mls. Emulsion recovery is approximately 80 to 90% when preparing volumes of 1 to 10 mls and approximately 50 to 60% when preparing volumes of <0.5 ml.

Materials:

1. **TiterMax®** #R-1 Research Adjuvant
2. Two 3.0 ml all-plastic syringes
3. One 18 gauge needle for withdrawing **TiterMax®** from the vial, or syringe without needle or positive displacement precision pipette if you open entire vial
4. One 18 gauge double hub emulsification needle (see Page 8)
5. Antigen in saline or other suitable fluid (typical dose range in mice is 15 to 125 µg/mouse)

Procedure:

NOTE: Prior to preparation of a **TiterMax®** water-in-oil emulsion, warm the **TiterMax®** to room temperature and vortex for 30 seconds. Make sure the **TiterMax®** is a homogeneous suspension of copolymer-coated microparticles before proceeding to emulsify by any method.

For 1 ml of a 50:50 water-in-oil emulsion you will need 0.5 ml of the aqueous antigen and 0.5 ml of **TiterMax®**.

1. After **TiterMax®** has been vortexed, load a syringe with 0.5 ml **TiterMax®**. Load the second syringe with 0.25 ml of antigen in aqueous medium. Set aside the other 0.25 ml of antigen. **NOTE:** It is important to add the aqueous antigen phase to the **TiterMax®** in at least 2 small volumes.
2. Connect the two syringes via an 18 gauge double hub emulsifying needle (Figure 1). Mix the **TiterMax®** with the antigen by forcing the materials back and forth through the needle for approximately 1 minute. **NOTE:** It is important to push the antigen into the **TiterMax®** syringe first, so that the aqueous phase enters the oil phase rather than vice versa. Hold the syringes carefully so that they do not come apart from the double hub needle during emulsification. The formation of a water-in-oil emulsion is signaled by a sudden increase in viscosity, (i.e., more force is required to move the material through the needle). **NOTE:** After approximately 1 minute a whipped-cream-like water-in-oil emulsion forms. Push all of the emulsion into one syringe and disconnect the empty syringe.
3. Load the empty syringe with the remaining 0.25 ml aqueous antigen solution. Reconnect the syringes and emulsify for another 30 to 60 seconds. **NOTE:** Again, first push the antigen into the water-in-oil emulsion. Care must be taken in holding the syringes together since the oil may lubricate and loosen the connection. That is why it is preferable to use a lock tip syringe. Push all of the emulsion into one syringe and disconnect the empty syringe. If necessary, load the syringe you have chosen for injecting animals.
4. To test for stability, place a drop of emulsion on water (see details on Page 7).

Precautions:

The syringes should be all-plastic. Plastic syringes with rubber pistons contain a lubricant which fails in the presence of **TiterMax®** and causes the syringes to stick. Use caution not to loosen the syringes from the double hub needle during emulsification. This will cause you to lose the emulsion.

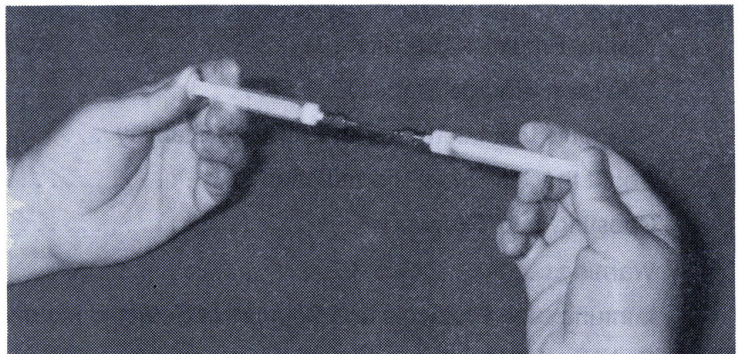


Figure 1: One ml of a 50:50 water-in-oil emulsion being prepared using a double hub emulsification needle and two all-plastic syringes.

Method 2: Kontes Pellet Pestle® Homogenizer

This method is suitable for emulsion volumes between 0.5 and 1 ml, but can be used with volumes as small as 0.1 ml. Recovery of emulsion is approximately 70 to 80%. The emulsion will be prepared directly in the syringe which will be used for immunization.

Materials:

1. TiterMax® #R-1 Research Adjuvant
2. Kontes Pellet Pestle® motor and suitable pestle, e.g. plastic pestle for larger volumes and wooden applicator stick for small volumes (see Page 8)
3. One 18 gauge needle for withdrawing TiterMax® from the vial, or syringe without needle or positive displacement precision pipette if you open entire vial
4. Syringe used for preparation of emulsion and immunization; piston removed; tip sealed with Parafilm®
5. Parafilm® for sealing tip of syringe
6. Antigen in saline or other suitable fluid (typical dose range in mice is 15 to 125 µg/mouse)

Procedure:

NOTE: Prior to preparation of a TiterMax® water-in-oil emulsion, warm the TiterMax® to room temperature and vortex for 30 seconds. Make sure the TiterMax® is a homogeneous suspension of copolymer-coated microparticles before proceeding to emulsify by any method.

For 0.5 ml of a 50:50 water-in-oil emulsion you will need 0.25 ml of the aqueous antigen and 0.25 ml TiterMax®.

1. After TiterMax® has been vortexed, load the Parafilm®-sealed syringe to be used for injecting animals with 0.25 ml TiterMax®. Add 0.25 ml of antigen in aqueous medium. **NOTE:** It is not necessary to add the aqueous antigen in small volumes when using this method.

2. Insert pestle and homogenize for approximately 1 minute. After approximately 1 minute a whipped-cream-like water-in-oil emulsion forms (Figure 2).

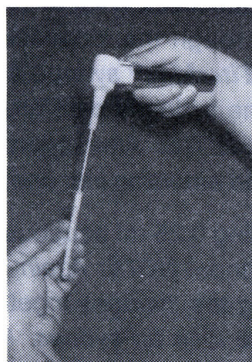


Figure 2: 0.5 ml of a 50:50 water-in-oil emulsion being prepared using the Kontes Pellet Pestle® motor, a wooden applicator stick and 1 ml all plastic syringe.

1. Remove piston from syringe
2. Seal syringe tip with Parafilm®
3. Add TiterMax® and antigen
4. Homogenize
5. Remove seal
6. Insert piston, add needle and inject

3. Remove the pestle from the syringe, scraping back into the syringe as much of the emulsion as possible. Replace piston of the syringe and put on appropriate needle for injecting animals. **NOTE:** Care must be taken when placing the piston back in the syringe. Add the piston slowly as you tap down the emulsion.
4. To test stability, place the pestle with a small amount of emulsion in water (see details on Page 7).

Precautions:

Be careful when adding the piston back to the syringe. The piston must be placed back into the syringe barrel slowly.

NOTE: Mechanical Homogenizers

TiterMax® forms excellent emulsions with many mechanical homogenizers (e.g., VirTis Hi-Speed or a Potter-Elvehjem with overhead stirrer or certain types of industrial homogenizers). However, such devices require large volumes of emulsion and are wasteful in that significant amounts cannot be readily transferred to syringes for injection. The above method using the Kontes Pellet Pestle® homogenizer which works in a plastic syringe has proven quite useful.

Method 3: Two-Syringe, 3-Way Stopcock

This method is suitable for emulsion volumes between 1 ml and 10 mls. Available 3-way stopcocks have larger bores than the 18 gauge double hub needles (Method 1) so that emulsification takes longer and the syringes connected to 3-way stopcocks are often more difficult to hold. Recovery of emulsion is approximately 70 to 80%.

Materials:

1. **TiterMax®** #R-1 Research Adjuvant
2. Two 3.0 ml all-plastic or siliconized glass syringes (preferably lock tip)
3. One 18 gauge needle for withdrawing **TiterMax®** from the vial, or syringe without needle or positive displacement precision pipette if you open entire vial
4. One 3-way plastic disposable or stainless steel reusable stopcock (see Page 8)
5. Antigen in saline or other suitable fluid (typical dose range in mice is 15 to 125 μ g/mouse)

Procedure:

NOTE: Prior to preparation of a **TiterMax®** water-in-oil emulsion, warm the **TiterMax®** to room temperature and vortex for 30 seconds. Make sure the **TiterMax®** is a homogeneous suspension of copolymer-coated microparticles before proceeding to emulsify by any method.

For 1 ml of a 50:50 water-in-oil emulsion you will need 0.5 ml of the aqueous antigen and 0.5 ml of **TiterMax®**

1. After **TiterMax®** has been vortexed, load a syringe with 0.5 ml **TiterMax®**. Load the second syringe with 0.25 ml of antigen in aqueous medium. Set aside the other 0.25 ml of antigen. **NOTE:** It is important to add the aqueous antigen phase to the **TiterMax®** in at least 2 small volumes.
2. Connect the two syringes via a 3-way stopcock (Figure 3). Mix the **TiterMax®** with the antigen by forcing the materials back and forth through the stopcock for approximately 1 minute. **NOTE:** It is important to push the antigen into the **TiterMax®** syringe first, so that the aqueous phase enters the oil phase rather than vice versa. Hold the syringes carefully so that they do not come apart from the 3-way stopcock during emulsification. **NOTE:** After approximately 1 minute a whipped-cream-like water-in-oil emulsion forms. Push all of the emulsion into one syringe and disconnect the empty syringe.
3. Load the empty syringe with the remaining 0.25 ml aqueous antigen solution. Reconnect the syringes and emulsify for another 30 to 60 seconds. **NOTE:** Again, first push the antigen into the water-in-oil emulsion. Care must be taken in holding the syringes together since the oil may lubricate and loosen the connection. It is preferable to use a lock tip syringe. Push all of the emulsion into one syringe. Disconnect the empty syringe and connect the syringe you have chosen for injecting for filling. Alternatively, simply disconnect the full syringe and add the appropriate needle for injecting animals.
4. To test stability, place a drop of emulsion on water (see details on Page 7).

Precautions:

The syringes should be siliconized glass or all-plastic. Plastic syringes with rubber pistons contain a lubricant which fails in the presence of **TiterMax®** and causes the syringes to stick. Use extreme caution during emulsification so that you do not loosen the syringes from the 3-way stopcock. This will cause you to lose the emulsion.

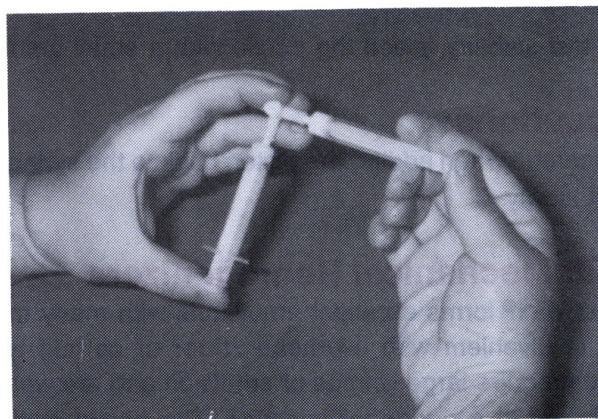


Figure 3: 1 ml of a 50:50 water-in-oil emulsion being prepared using the 3-way stopcock and two all-plastic syringes.

Method 4: One Syringe, Blunt Needle

This method is useful for volumes less than 0.5 ml and has been used with volumes as low as .05 ml final emulsion volume. Recovery of emulsion is approximately 50 to 75%.

Materials:

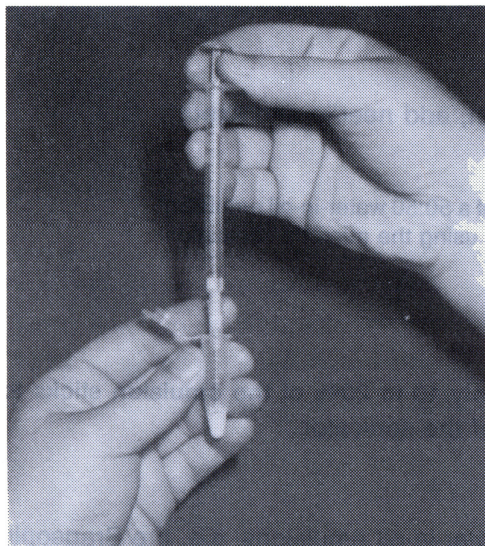
1. **TiterMax®** #R-1 Research Adjuvant
2. 1 ml all-plastic syringe
3. 18 gauge \times 1.5 inch blunt needle for emulsifying
4. 1.5 ml conical bottom plastic centrifuge tube
5. One 18 gauge needle for withdrawing **TiterMax®** from the vial, or syringe without needle or positive displacement precision pipette if you open entire vial
6. Antigen in saline or other suitable fluid (typical dose range in mice is 15 to 125 μ g/mouse)

Procedure:

NOTE: Prior to preparation of a **TiterMax®** water-in-oil emulsion, warm the **TiterMax®** to room temperature and vortex for 30 seconds. Make sure the **TiterMax®** is a homogeneous suspension of copolymer-coated microparticles before proceeding to emulsify by any method.

For 200 μ l of a 50:50 water-in-oil emulsion you will need 100 μ l of the aqueous antigen and 100 μ l of **TiterMax®**.

1. Grasp the pointed end of the needle with pliers and gently bend it back and forth until the tip breaks off producing a 1 inch blunt end needle. Attach the blunt 18 gauge needle to a 1 ml all-plastic syringe.
2. Add 100 μ l of **TiterMax®** adjuvant to the 1.5 ml conical bottom centrifuge tube. Add 50 μ l of your antigen solution. The antigen-adjuvant mixture is drawn into the syringe and expressed back into the tube several times until a thick white emulsion forms. Add the remaining 50 μ l of your antigen solution and repeat the process (Figure 4). **NOTE:** Certain technical points are important. The air drawn into the syringe during the process does not impede the emulsification process. In approximately 1 minute, the entire material will be transformed into a water-in-oil emulsion. If one is careful not to smear the material on the sides of the tube, it can be drawn almost quantitatively into the syringe (using the 18 gauge needle). If you get emulsion on the sides of the tube, centrifuge at low speed (100 \times g) for 2 minutes to pellet the emulsion. Remove the blunt needle and replace with a suitable needle for injecting.
3. To test stability, place a tiny drop of emulsion on or in water (see details on Page 7).



1. **Draw emulsion forcefully** into syringe
2. **Express slowly** to avoid splattering; hold needle centered or against side of tube
3. Continue until a water-in-oil emulsion forms
4. If emulsion sticks to the walls of the tube, force it to the bottom by gentle centrifugation
5. Replace blunt 18 gauge needle with a suitable one for injection.

Figure 4: 0.2 ml of a 50:50 water-in-oil emulsion being prepared using the one-syringe blunt needle and conical-bottom plastic tube.

Method 5: Emulsifying in Syringe Using Sonication

This method is suitable for emulsion volumes between 0.5 ml and 2 ml. Recovery of emulsion is approximately 80 to 90%.

Materials:

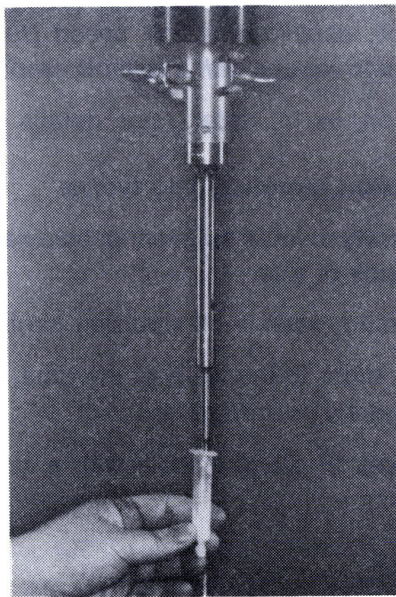
1. **TiterMax**[®] #R-1 Research Adjuvant
2. One 18 gauge needle for withdrawing **TiterMax**[®] from the vial, or syringe without needle or positive displacement precision pipette if you open entire vial
3. One syringe for preparation of emulsion and immunization; piston removed; tip sealed with Parafilm[®]
4. Parafilm[®] for sealing tip of syringe
5. Antigen in saline or other suitable fluid (typical dose range in mice is 15 to 125 $\mu\text{g}/\text{mouse}$)
6. Sonic dismembrator with microtip

Procedure:

NOTE: Prior to preparation of a **TiterMax**[®] water-in-oil emulsion, warm the **TiterMax**[®] to room temperature and vortex for 30 seconds. Make sure the **TiterMax**[®] is a homogeneous suspension of copolymer-coated microparticles before proceeding to emulsify by any method.

For 1.0 ml of a 50:50 water-in-oil emulsion you will need 0.5 ml of the aqueous antigen and 0.5 ml of **TiterMax**[®].

1. Carefully seal the tip of the syringe with Parafilm[®]. After **TiterMax**[®] has been vortexed, load a 2.0 ml syringe with 0.5 ml **TiterMax**[®]. Add 0.5 ml of antigen in aqueous medium. **NOTE:** It is not necessary to add the aqueous antigen in small volumes when using this method.
2. Place microtip of sonicator into syringe and turn on power. After approximately 35 to 45 seconds a whipped-cream-like water-in-oil emulsion forms (Figure 5). **NOTE:** Care must be taken to seal the syringe. Push all of the emulsion together by tapping as you insert the piston.
3. To test stability, place a small amount of emulsion on or in water (see details on Page 7).



1. Remove piston from syringe
2. Seal syringe tip with Parafilm[®]
3. Add **TiterMax**[®] and antigen
4. Sonicate
5. Remove seal
6. Insert piston, add needle and inject

Figure 5: 1 ml of a 50:50 water-in-oil emulsion being prepared using the microtip sonication method.

Precautions:

Approximately 10 to 20% of the emulsion sticks to the microtip of the sonicator.

NOTE: Sonicators

Other types of sonicators have also been used to prepare water-in-oil emulsions, e.g. the Bransonic 32. Using the Bransonic takes several minutes longer and requires extra care to ensure the syringe is sealed and protected from the water.

Stability of the TiterMax® #R-1 Water-in-Oil Emulsion

To test whether your TiterMax® emulsion is ready to use, expel a tiny drop onto the surface of water. It should expel from the syringe with a consistency similar to whipped cream and should hold together on the surface of water (Figure 6). If you are preparing a small volume of emulsion, you may touch the tip of a pipette or applicator stick to your emulsion preparation and submerge it into water (Figure 7). Either way, the emulsion should hold together. **NOTE:** In Figure 6 one can see the strands of a 50:50 water-in-oil emulsion on the surface of water expelled from a 1 ml syringe through a 27 gauge needle. In Figure 7, note the strips of a 50:50 water-in-oil emulsion coating the wooden applicator stick. In the event that the emulsion disperses on or in the water, reconnect the syringe (or repeat small volume procedure) and emulsify for another minute.

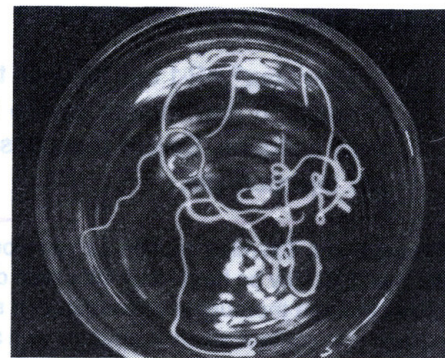


Figure 6

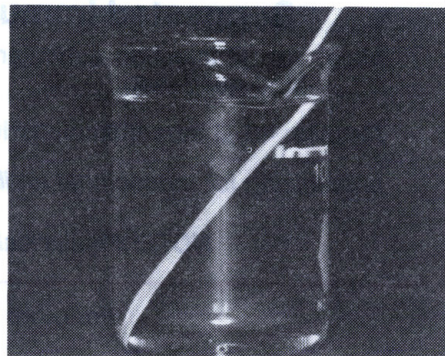


Figure 7

Stability of the Emulsion after Prolonged Storage

A 50:50 water-in-oil emulsion can usually be stored at room temperature, 4°C, -20°C or -70°C for as long as your antigen is stable. Upon storage, approximately 20% of the oil will dissociate from the emulsion. You may leave the emulsion in a syringe and simply reemulsify when you are ready to use again for injecting. The stability of your antigen must be considered to do this. TiterMax® emulsions have been repeatedly frozen and thawed or left at room temperature for several weeks.

Reagents that May Interfere with Emulsification or Stability

Immunogens which contain high concentrations of surfactants or other materials may interfere with emulsification. We have found that SDS, which may be present in acrylamide gels, in concentrations > 1% or urea in concentrations > 1.0 M significantly reduces the emulsifying capacity of TiterMax®. Other similar materials are likely to have the same effect. Some surface active agents serve as demulsifying agents which break emulsions. The modern emulsifiers and microparticulate stabilizer of TiterMax® are able to overcome the effects of most such agents present in moderate quantities.

About TiterMax® #R-1 Formulation

Each vial of TiterMax® contains sufficient volume to easily recover 500 µl. TiterMax® is formulated with squalene, a metabolizable oil. The active ingredient is a synthetic block copolymer, CRL-8941, bonded to the surface of silica particles. These copolymer-coated microparticles act as stabilizers for the water-in-oil emulsion. TiterMax® also contains a surfactant.

Good immune responses have been achieved with ratios of water to TiterMax® of 50 to 90%, but the 50:50 water-in-oil emulsion is usually optimal. As you increase the water content of the water-in-oil emulsion, remember that you are decreasing the amount of the active ingredient, CRL-8941, in the oil phase. The investigator must carefully titrate dose and emulsion formulation for each individual antigen. We have been successful in producing high antibody titers with an antigen concentration range of 15 to 125 µg/dose, depending on the antigen.

Boosting with TiterMax® #R-1

Since TiterMax® produces high antibody titers without boosting, a single injection may produce sufficient antibody for your needs. Be aware that injecting a depot of antigen into an animal with high antibody titers can elicit a local Arthus reaction. If primary titers are high, boost with half or less of the immunizing dose of TiterMax® or you may boost with antigen in saline to avoid any local Arthus reaction. We have been successful in boosting with either TiterMax® or soluble antigen.

WARNING:

TiterMax[®] adjuvants can be used to induce high antibody titers with only mild local inflammation. However, it may induce severe local reactions if used in excessive doses or with inherently toxic antigens. Severe reactions are dose dependent and rarely occur with injections $\leq 100 \mu\text{l}$ per injection site.

Precautions: Exercise caution as you would with any potent biological agent to avoid accidental contact, especially via injection. May be harmful by inhalation due to aerosolization, ingestion or skin absorption. The toxicological properties have not been thoroughly investigated. If swallowed, wash out mouth with water provided the person is conscious. In case of skin contact, wash thoroughly with soap and water. Remove contaminated clothing and shoes. If inhaled, remove the person to fresh air. If breathing becomes difficult, call a physician. In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

For more information or to place an order contact:

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Caution: **TiterMax[®] Classic** and **TiterMax[®] Gold** are adjuvants for investigational use only in laboratory research animals, or tests in vitro. Not for use in humans.

Emulsification Equipment:

*All of the following materials are available as a convenience for our **TiterMax[®]** customers. Please specify the item by name, product code and the quantity desired.*

Product Codes:

All plastic 1 cc syringe.....	#M-1-1.0
All plastic 3.0 cc syringe.....	#M-1-3.0
Plastic disposable 3-way stopcocks	#M-2
Double hub emulsification needles	#M-3
Kontes Pellet Pestle [®] Motor	#M-4