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# Product Information

**cGMP Enzyme Immunoassay Kit**

Catalog Number **CG201**
Storage Temperature –20 °C

## TECHNICAL BULLETIN

### Product Description

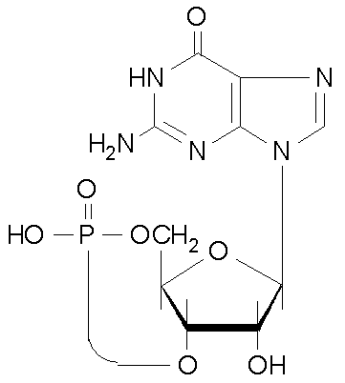
The EIA cyclic GMP kit is a competitive immunoassay for the quantitative determination of cyclic GMP in samples. The kit uses a polyclonal antibody to cGMP to bind, in a competitive manner, the cGMP in the sample or an alkaline phosphatase molecule that has cGMP covalently attached to it. Samples or standards, alkaline phosphatase conjugate, and antibody are simultaneously incubated at room temperature in a secondary antibody coated multiwell plate. The excess reagents are then washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a multiwell plate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of cGMP in either the standards or samples. The measured optical density is used to calculate the concentration of cGMP. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard or Tijssen.[1,2]

The cGMP EIA may be used to assay cGMP samples from a wide range of sources. Samples diluted sufficiently (>1:10) can be read directly from the standard curve. Samples containing rabbit IgG may interfere with the assay. Samples with very low levels of cGMP may be acetylated. Acetylation of the samples increases the sensitivity of the assay.

Guanosine 3′,5′-cyclic monophosphate (cyclic GMP, cGMP) was identified in 1963.[3] It has been shown to be present at levels typically 10 to 100-fold lower than cAMP in most tissues and is formed by the action of the enzyme guanylate cyclase on GTP. It is involved in a number of important biological reactions. Some hormones, such as acetylcholine, insulin, and oxytocin, as well as certain other chemicals like serotonin and histamine cause an increase in cGMP levels.[4,5]

Stimulators of guanylate cyclase such as the vasodilators nitroprusside, nitroglycerin, sodium nitrate, and nitric oxide (NO) also stimulate cGMP levels.[6] Peptides, such as atrial natriuretic peptide (ANP) that relax smooth muscle, also increase cGMP concentrations.[7] cGMP has been confirmed as a second messenger for ANP.[8] NO can be synthesized from L-arginine and diffuse through cell membranes.[9,10] The interaction of NO with guanylate cyclase allows cGMP to act as a third messenger in some cells.[11]

Cyclic GMP




### Components

Sufficient reagents are supplied for 96 assays

| | |
|---|---|
| Goat Anti-Rabbit IgG Coated 96 Well Multiwell Plate - break-apart strips coated with goat antibody specific to rabbit IgG (Catalog Number M3683) | 1 each |
| cGMP-Alkaline Phosphatase Conjugate A blue solution of alkaline phosphatase conjugated with cGMP (Catalog Number C6726) | 5 ml |
| cGMP EIA Antibody Rabbit Anti-cGMP A yellow solution of a polyclonal rabbit antibody to cGMP (Catalog Number C6476) | 5 ml |

Assay Buffer 2 30 ml
A buffer containing proteins, detergents, and sodium azide as preservative.
(Catalog Number A5219)

Wash Buffer Concentrate 30 ml
Tris buffered saline containing detergents and sodium azide as preservative.
(Catalog Number W1265)

Cyclic GMP Standard 0.5 ml
A solution of 5,000 pmole/ml cGMP
(Catalog Number C6851)

*p*-Nitrophenyl Phosphate Substrate Solution 20 ml
A ready-to-use solution of *p*-nitrophenyl phosphate in buffer. (Catalog Number N7408)

Stop Solution 5 ml
A solution of trisodium phosphate in water
Keep tightly capped. Caution: caustic
(Catalog Number S2436)

Triethylamine 2 ml
Caution: lachrymator, harmful vapor, flammable
(Catalog Number T7441)

Acetic Anhydride 1 ml
Caution: lachrymator, corrosive, flammable
(Catalog Number A5344)

Plate Sealer 1 each
(Catalog Number P2107)

**Equipment and Reagents Required but Not Provided**

- Deionized or distilled water. No difference in assay results is seen with distilled water.
- Precision pipettes for volumes between 5 µl and 1,000 µl
- Repeater pipettes for dispensing 50 µl and 200 µl
- Disposable beakers for diluting buffer concentrates
- Graduated cylinders
- A multiwell plate shaker
- Adsorbent paper for blotting
- Multiwell plate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
- 5 Cycle Log-Log Paper

**Precautions and Disclaimer**

This product is for R&D use only, not for drug, household, diagnostic, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Caution: Some components of this kit contain chemicals that are acidic, caustic, lachrymators, corrosive, and flammable. Use with caution and wear suitable protection. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.

**Preparation Instructions**

Reagent Preparation

Note: Standards can be made up in either glass or polypropylene tubes. Avoid polystyrene tubes.

1. cGMP Standard (Non-Acetylated Version) – Allow the Cyclic GMP Standard (5,000 pmole/ml) to warm to room temperature. Label six 12 × 75 mm tubes 1 through 6. Pipette 900 µl of Assay Buffer 2 into tube 1 and 800 µl into tubes 2–6. Add 100 µl of the 5,000 pmole/ml Cyclic GMP Standard to tube 1. Vortex thoroughly. Add 200 µl of tube 1 to tube 2 and vortex thoroughly. Continue this for tubes 3 through 6. The concentration of cGMP in tubes 1 through 6 will be 500, 100, 20, 4, 0.8, and 0.16 pmole/ml, respectively. Diluted standards should be used within 60 minutes of preparation.

2. Acetylation Reagent – Prepare the Acetylation Reagent by adding 0.5 ml of acetic anhydride to 1 ml of triethylamine. Use the prepared reagent within 60 minutes of preparation.

3. cGMP Standard (Acetylated Version) – Allow the Cyclic GMP Standard (5,000 pmole/ml) to warm to room temperature. Label five 12 × 75 mm tubes 1 through 5. Pipette 980 µl of Assay Buffer 2 into tube 1 and 800 µl of Assay Buffer 2 into tubes 2–5. Add 20 µl of the 5,000 pmole/ml Cyclic GMP Standard to tube 1. Vortex thoroughly. Add 200 µl of tube 1 to tube 2 and vortex thoroughly. Continue this for tubes 3 through 5.

   Label one 12 × 17 mm tube as the Zero Standard/ NSB tube. Pipette 1 ml of Assay Buffer 2 into this tube for use in Assay Procedure, step 3. The concentration of cGMP in tubes 1 through 5 will be 100, 20, 4, 0.8, and 0.16 pmole/ml, respectively.

Acetylate all standards and samples by adding 10 µl of the Acetylation Reagent for each 200 µl of standard or sample. Add the reagent directly to the samples and vortex for 2 seconds. Add 50 µl of the Acetylation Reagent to the Zero Standard/NSB tube and use in Assay Procedure, step 3 (Failure to acetylate the NSB and Zero will result in inaccurate B/Bo values). Use the acetylated standards or samples within 30 minutes.

4. 1× Wash Buffer – Prepare 1× Wash Buffer by diluting 10 ml of the Wash Buffer Concentrate with 90 ml of deionized water. This can be stored at room temperature for 3 months.

Sample Handling

The cGMP enzyme immunoassay is compatible with cGMP samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer 2 (>1:10) can be read directly from the standard curve. **Samples containing rabbit IgG may interfere with the assay.**

If samples with very low levels of cGMP are to be measured, reagents are provided to acetylate samples and standards. Acetylation of the samples increases the sensitivity of the assay. Please refer to references 15–21 for further methods of extraction of cGMP from samples.

Urine samples may be used in the assay directly by dilution in the range of 1:100 to 1:1,000 in Assay Buffer 2. Plasma samples should be drawn in tubes containing EDTA. EDTA chelates calcium and will stop phosphodiesterase activity. The plasma collected should be assayed immediately or frozen below –20 °C. 2 ml of 95% ethanol are added to 1 ml of the collected plasma. Vortex for 15 seconds and let sit at room temperature for 5 minutes. Centrifuge for 10 minutes at 600 × *g* at room temperature. Decant the supernatant into a clean tube. These samples should be dried down, reconstituted in Assay Buffer 2, and then used directly in the assay.

Tissue samples should be rapidly frozen in liquid nitrogen. Grind the frozen tissue to a fine powder under liquid nitrogen in a stainless steel mortar. Weigh the frozen tissue and homogenize in 10 volumes of cold 5% TCA in a glass-PTFE tissue grinder. Centrifuge at 600 × *g* for 10 minutes. Extract the supernatants with 3 volumes of water-saturated ether. Dry the aqueous extracts and run the reconstituted samples directly in the assay.

Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture medium instead of Assay Buffer 2. There will be a change in binding associated with running the standards and samples in the medium. Users should only use standard curves generated in medium or buffer to calculate concentrations of cGMP in the appropriate matrix.

For cells adhering to glass or plastic, add 0.1 M HCl to the samples and let sit for 10 minutes. Take off the 0.1 M HCl supernatant and centrifuge at 600 × *g* for 10 minutes. These samples may be assayed directly in our Direct cGMP kit, Catalog Number CG200, or dried down prior to reconstitution in Assay Buffer 2 and running in this kit.

**Storage/Stability**

The kit ships on wet ice and storage at –20 °C is recommended.

**Procedure**

Do not mix components from different kit lots.

The activity of the alkaline phosphatase conjugate is dependent on the presence of $Mg^{2+}$ and $Zn^{2+}$ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.

The performance of this kit has been tested with a variety of samples; however, it is possible that high levels of interfering substances may cause variation in assay results.

Allow all reagents to warm to room temperature for at least 30 minutes before opening.

Standards can be made up in either glass or polypropylene tubes. Avoid polystyrene tubes.

Keep unused plate strips sealed in bag with desiccant.

Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard, and reagent.

Pipette standards and samples to the bottom of the wells.

Add the reagents to the side of the well to avoid contamination.

This kit uses break-apart multiwell strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 2–8 °C in the sealed foil bag. The wells should be used in the frame provided.

The cyclic GMP Standard provided, Catalog Number C6851, is supplied in ethanolic buffer at a pH optimized to maintain cGMP integrity. Care should be taken in handling this material because of the known and unknown effects of cGMP on biological tissue.

Care must be taken to **minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipette tips and other items that are used in the assay with bare hands.

Assay Procedure

Allow all reagents to warm to room temperature for at least 30 minutes before opening.

All standards and samples should be run in duplicate.

If the Acetylated Version of the kit is to be run, acetylate all standards and samples by adding 10 µl of the Acetylation Reagent for each 200 µl of standard or sample. Add 50 µl of the Acetylation Reagent to the Zero Standard/NSB tube (refer to Reagent Preparation, step 3) and use in steps 3 and 6 in this procedure. Add the reagent directly to the samples and vortex for 2 seconds. Use the acetylated standards or samples within 30 minutes.

1. Determine the number of wells to be used and put any remaining wells with the desiccant back into the foil pouch and seal. Store unused wells at 2–8 °C.
2. Pipette 100 µl of Assay Buffer 2 into the NSB and the Bo (0 pmole/ml Standard) wells.
3. Pipette 100 µl of the prepared Standards into the appropriate wells.
4. Pipette 100 µl of the samples into the appropriate wells.
5. Pipette 50 µl of Assay Buffer 2 into the NSB wells.
6. Pipette 50 µl of blue cGMP-Alkaline Phosphatase Conjugate into each well except the TA and Blank wells.
7. Pipette 50 µl of yellow cGMP EIA Antibody into each well, except the Blank, TA (total activity), and NSB (non specific binding) wells.
   Note: Every well used should be **Green** in color except the NSB wells which should be **Blue.** The Blank and TA wells are empty at this point and have no color.
8. Incubate the plate at room temperature for 2 hours on a plate shaker at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.
9. Empty the contents of the wells and wash by adding 200 µl of 1× Wash Buffer to every well. Repeat the wash 2 more times for a total of **3** washes.
10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
    Note: Prior to addition of substrate, ensure there is no residual Wash Buffer in the wells. Any remaining Wash Buffer in the wells may cause variation in assay results.
11. Add 5 µl of the blue cGMP-Alkaline Phosphatase Conjugate to the TA wells.
12. Add 200 µl of the *p*-Nitrophenyl Phosphate Substrate Solution to every well. Incubate at room temperature for 1 hour without shaking.
13. Add 50 µl of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader cannot be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

**Results**

Several options are available for the calculation of the concentration of cGMP in the samples. It is recommended the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic curve fitting program such as "AssayZap" (www.biosoft.com). If this type of data reduction software is not readily available, the concentration of cGMP can be calculated as follows:

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD =
   Average Bound OD – Average NSB OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

   $$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Using the Logit-Log paper plot Percent Bound (B/Bo) versus Concentration of cGMP for the standards. Approximate a straight line through the points. The concentration of cGMP in the unknowns can be determined by interpolation.

**Product Profile**

Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from other assays.

Non-Acetylated Version

| Sample | Net OD | Percent Bound | **cGMP (pmole/ml)** |
|---|---|---|---|
| Blank OD | (0.176) | | |
| TA | 0.296 | | |
| NSB | 0.000 | 0% | |
| Bo | 0.519 | 100% | **0** |
| S1 | 0.010 | 1.93% | **500** |
| S2 | 0.047 | 9.06% | **100** |
| S3 | 0.156 | 30.06% | **20** |
| S4 | 0.352 | 67.82% | **4** |
| S5 | 0.455 | 87.67% | **0.8** |
| S6 | 0.507 | 97.69% | **0.16** |
| Unknown1 | 0.300 | 58.94% | **6.05** |
| Unknown 2 | 0.098 | 18.88% | **39.29** |

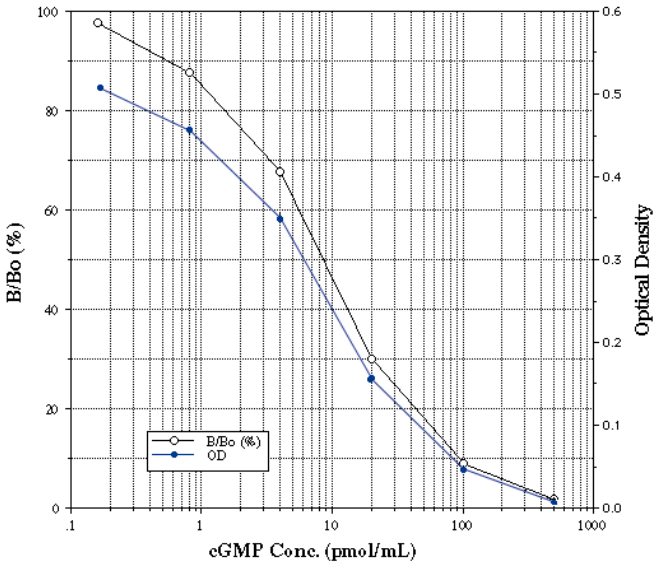
Acetylated Version

| Sample | Net OD | Percent Bound | **cGMP (pmole/ml)** |
|---|---|---|---|
| Blank OD | (0.146) | | |
| TA | 0.320 | | |
| NSB | 0.002 | 0% | |
| Bo | 0.266 | 100% | **0** |
| S1 | 0.003 | 1.13% | **100** |
| S2 | 0.018 | 6.77% | **20** |
| S3 | 0.062 | 23.31% | **4** |
| S4 | 0.149 | 56.02% | **0.8** |
| S5 | 0.223 | 83.84% | **0.16** |
| S6 | – | – | **–** |
| Unknown1 | 0.130 | 48.87% | **1.12** |
| Unknown 2 | 0.010 | 3.76% | **33.87** |

Typical Standard Curves

These curves **must not** be used to calculate cGMP concentrations; each user must run a standard curve for each plate and version used.

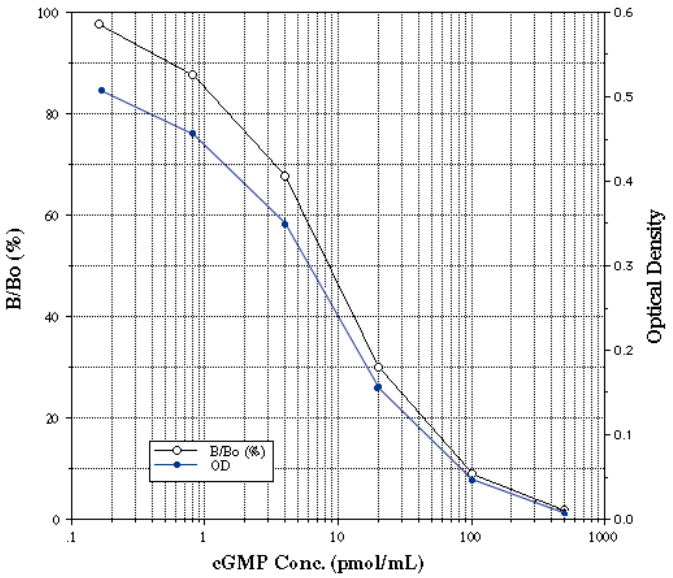
Non-Acetylated Version




Typical Quality Control Parameters

| | | |
|---|---|---|
| Total Activity Added | = | 0.296 × 10 = 2.96 |
| %NSB | = | 0.0% |
| %Bo/TA | = | 17.6% |
| Quality of Fit | = | 0.99995 |
| | | |
| 20% Intercept | = | 36.5 pmole/ml |
| 50% Intercept | = | 8.40 pmole/ml |
| 80% Intercept | = | 1.90 pmole/ml |

Acetylated Version




Typical Quality Control Parameters

| | | |
|---|---|---|
| Total Activity Added | = | 0.320 × 10 = 3.20 |
| %NSB | = | 0.0% |
| %Bo/TA | = | 8.3% |
| Quality of Fit | = | 0.99999 |
| | | |
| 20% Intercept | = | 5.1 pmole/ml |
| 50% Intercept | = | 1.1 pmole/ml |
| 80% Intercept | = | 0.2 pmole/ml |

**Specificity**

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.[19]

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard 6 in the Non-Acetylated or with Standard 5 in the Acetylated version. The detection limit was determined as the concentration of cGMP measured at two (2) standard deviations from the zero along the standard curve.

Non-Acetylated Version

Mean OD for Bo = 0.397 ± 0.013 (3.3%). Mean OD for Standard 6 = 0.385 ± 0.012 (3.2%).
Delta Optical Density (0–0.16 pmole/ml) = 0.397 – 0.385 = 0.012. 2 SD's of Bo = 0.026
Sensitivity = $\frac{0.026}{0.012} \times 0.16$ pmole/ml = **0.37 pmole/ml**

Acetylated Version

Mean OD for Bo = 0.280 ± 0.008 (2.8%). Mean OD for Standard 5 = 0.251 ± 0.013 (5.3%).
Delta Optical Density (0–0.16 pmole/ml) = 0.280 – 0.251 = 0.029. 2 SD's of Bo = 0.016
Sensitivity = $\frac{0.016}{0.029} \times 0.16$ pmole/ml = **0.088 pmole/ml**

Linearity

Non-Acetylated Version

A sample containing 800 pmole/ml cGMP was serially diluted 8 times 1:2 in Assay Buffer 2 and measured in the assay. The data was plotted graphically as actual cGMP concentration versus measured cGMP concentration. The line obtained had a slope of 1.060 with a correlation coefficient of 0.998.

Acetylated Version

A sample containing 5.79 pmole/ml cGMP was serially diluted 3 times 1:2 in Assay Buffer 2 and measured in the Acetylated version of the assay. The data was plotted graphically as actual cGMP concentration versus measured cGMP concentration. The line obtained had a slope of 0.90303 with a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium, and high concentrations of cGMP and running these samples multiple times ($n \geq 8$) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium, and high concentrations of cGMP in multiple assays (n = 8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of cGMP determined in these assays as calculated by a 4 parameter logistic curve fitting program.

Non-Acetylated Version

| | cGMP (pmole/ml) | Intra Assay (%CV) | Inter Assay (%CV) |
|---|---|---|---|
| Low | 1.5 | 5.2 | |
| Medium | 16.6 | 4.0 | |
| High | 480.8 | 7.6 | |
| Low | 1.8 | | 13.7 |
| Medium | 16.9 | | 3.5 |
| High | 359.0 | | 5.0 |

Acetylated Version

| | cGMP (pmole/ml) | Intra Assay (%CV) | Inter Assay (%CV) |
|---|---|---|---|
| Low | 0.54 | 6.5 | |
| Medium | 1.50 | 4.6 | |
| High | 6.81 | 4.5 | |
| Low | 0.70 | | 5.9 |
| Medium | 1.96 | | 6.2 |
| High | 8.61 | | 6.8 |

Cross Reactivities

The cross reactivities for a number of related compounds were determined by dissolving the cross reactant (purity checked by NMR and other analytical methods) in Assay Buffer 2 at concentrations from 10,000 to 10 pmole/ml. These samples were then measured in the cGMP assay, and the measured cGMP concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

| Compound | Cross Reactivity |
|---|---|
| cGMP | 100% |
| GMP | <0.001% |
| GTP | <0.001% |
| cAMP | <0.001% |
| AMP | <0.001% |
| ATP | <0.001% |
| cUMP | <0.001% |
| CTP | <0.001% |

Sample Recoveries

cGMP concentrations were measured in a variety of different samples including tissue culture media and human saliva, serum, and urine. For all of the samples, cGMP was spiked into the undiluted samples, which were diluted with Assay Buffer 2 and then assayed in the kit. The following results were obtained:

Non-Acetylated Version

| Sample | % Recovery | Recommended Dilution* |
|---|---|---|
| Tissue Culture Media | 101.7 | 1:100 |
| Human Saliva | 102.9 | 1:10 |
| Human Serum | 101.3 | 1:10-1:100 |
| Human Heparin Plasma | 104.4 | 1:10 |
| Human EDTA Plasma | 115.0 | 1:10-1:100 |
| Human Urine | 97.7 | 1:100-1:1000 |

Acetylated Version

| Sample | % Recovery | Recommended Dilution* |
|---|---|---|
| Tissue Culture Media | 95.8 | Undiluted |
| Human Saliva | | |
| Human Serum | | Not Recommended |
| Human Heparin Plasma | | |
| Human EDTA Plasma | 93.6 | ≥ 1:2 |
| Human Urine | | |

* See Sample Handling instructions on Page 3 for Details

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KAA,LPG,MAM 04/10-1